REVIEW ARTICLE

Emerging evidence for the modulation of exocytosis by signalling lipids

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Membrane fusion is a key event in exocytosis of neurotransmitters and hormones stored in intracellular vesicles. In this process, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins are essential components of the exocytotic molecular machinery, while lipids have been seen traditionally as structural elements. However, the so-called signalling lipids, such as sphingosine and arachidonic acid, interact with SNAREs and directly modulate the frequency and mode of fusion events. Interestingly, recent work has proved that the sphingosine analogue FTY-720, used in the treatment of multiple sclerosis, mimics the effects of signalling lipids. In the present Review, we discuss recent investigations suggesting that endogenous signalling lipids and synthetic analogues can modulate important physiological aspects of secretion, such as quantal release, vesicle recruitment into active sites, vesicle transport and even organelle fusion in the cytosol. Therefore, these compounds are far from being merely structural components of cellular membranes.

Keywords: exocytosis; signalling lipids; SNARE proteins

The fusion of the vesicles containing neurotransmitters and hormones with the plasma membrane to release their content during the process of exocytosis is a key event underlying the function of the neuronal and endocrine systems. In essence, this is a multisequential process involving the cytoskeletal-mediated transport of vesicles [1,2], their docking and final release of neurotransmitters through the interactions of soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins and the lipids constituting the vesicular and plasma membrane [3,4].

The lipids within the membranes were initially assumed to play a passive role, but recent results indicate that a class known as signalling lipids directly modulate SNARE function, and may play important roles in the physiology of neurosecretion [5–7].

Signalling lipids that modulate SNARE function include arachidonic acid (AA) and sphingosine. AA is generated from a variety of phospholipid molecules by phospholipase-A2 or diacylglycerol lipase whereas sphingosine is produced from sphingolipids. AA primarily seems to target t-SNARE protein syntaxin-1 [8,9], which is anchored to the plasma membrane, whereas sphingosine interacts with synaptobrevin, which is the complementary v-SNARE anchored to the vesicle. Both AA and sphingosine seem to enhance exocytosis

of both neurotransmitters and hormones by promoting formation of SNARE complexes [10].

Interestingly, a structural analogue of sphingosine of fungal origin, FTY-720, also known as Fingolimod, has been approved for the oral treatment of multiple sclerosis [11], one of the most frequent disorders of the CNS. In this case, this substance is phosphorylated and binds to sphingosine-1-phosphate receptors, causing lymphocyte egress and immunosuppression, therefore being effective for the palliation of this CNS inflammatory syndrome [12,13]. Nevertheless, this drug, as it happens with signalling lipids, also enhances SNARE complex formation and promotes the release of neurotransmitters in neuroendocrine cellular models [14]. Here, we review the molecular mechanisms and the exocytotic steps regulated by signalling lipids and related molecules affecting the secretory activity of neuronal and neuroendocrine cells.

The traditional and new role of lipids in exocytotic membrane fusion

Neurotransmitters and hormones are stored in specialized vesicles. The release of these active substances requires the fusion of the membrane forming these vesicles with the plasma membrane during exocytosis [15–17]. The process initiates with the formation of a structure called the fusion pore, formed with lipids from the opposite membranes [6,18,19].

As the formation of the fusion pore requires the active reorganization of lipids to overcome energy barriers, the actual 'proteocentric' vision claimed catalytic proteins as sculptors of the lipid bilayers, giving the lipids a mere passive structural role. Since lipid bilayers need to adopt curved shapes during membrane fusion, either proteins or lipids could help in the spontaneous bending, therefore the shape of the lipids forming the fusing leaflets have an important role in the facilitation of exocytosis [20]. In this sense, conic shape lipids with larger or smaller heads comparing with its inner fatty acid chains will facilitate membrane curvature and in consequence there is a large number of evidences supporting this 'shaping' role [7,20–23], especially for lysophospholipids regulating the secretion in neuroendocrine cells (see Fig. 1) [24,25].

Lipids can additionally influence exocytosis by aggregating into specific microdomains that recruit proteins required for neurosecretion. In this sense, it is well-established that phosphatidyl inositol 4,5-biphosphate (PIP2) is a specific requirement for exocytosis (Fig. 1) [26], being recruited in secretory sites by intracellular calcium elevations during cell stimulation [27]. Today, these initial studies have been supported by recent studies proving that PIP2 coordinates the translocation of secretory vesicles to their docking sites on the plasma membrane in a Cdc42-dependent manner [28,29]. In that way, forms clusters that, in addition to nucleation of the formation of F-actin bundles, also interact with SNARE proteins [30], and in consequence act as a beacon for vesicle guidance to active secretory sites (Fig. 1).

In addition, other membrane constituents, such as cholesterol, are essential for maintaining the heterogeneities in the plasma membrane that accumulate secretory proteins such as syntaxin-1 in well-defined clusters within so called lipid rafts [31–35].

Finally, lipids are incorporated into secretory proteins to modify them and affect in that way either the location or the activity. This post-translation modification consists frequently in an acylation by the incorporation of palmitate, a saturated 16-carbon fatty acid, into cysteine residues [36], and the major target in neuroendocrine cells is the SNARE protein synaptosomal-associated protein of 25 kDa (SNAP-25) [37–39]. Palmitoylation of SNAP-25 in four central residues (Fig. 1)[38], is likely to enhance the clustering of SNAP-25 in cholesterol and sphingomyelin rich lipid rafts and in that way may be a cohesive factor in the formation of exocytotic active sites [33,40,41]. The exact role of SNAP-25 palmitoylation is unclear, since some studies indicate that it is a major factor supporting the secretory activity of this protein transmitting to the fusing membranes the proper forces generated during SNARE complex assembly or zippering [42], whilst others suggest that the palmitoylation serves a more conventional role in membrane anchoring [37].

It is important to mention that SNAP-25 is not the only protein relevant for exocytosis that is pamitoylated, as synaptobrevin 2, present in the vesicular membrane could be modified by palmitic acylation during brain development, as this modification is only found in adults and not in embryonic rats [43]. In addition, synaptotagmin 1, an essential calcium sensor [44], is palmitoylated in five residues near the transmembrane domain [38]. Finally, cystein string protein (CSP), a molecular chaperone helping in protein folding [45], is heavily palmitoylated in 14 cystein residues and it has been found to be important for the secretory process in neuronal and endocrine models [46–48].

As could be derived from the multiple roles assumed by lipids as mentioned above, their function in exocytosis is far more complex than the deduced from being the basic structural elements forming membranes, and this is further evidenced with recent data on the direct modulation of the secretory machinery by signalling lipids.
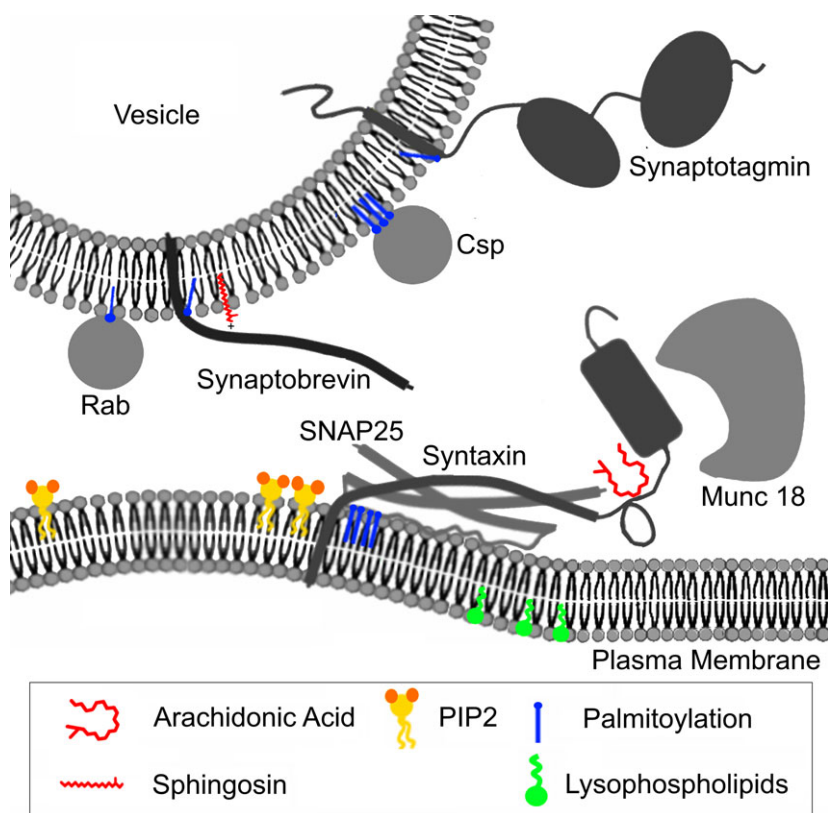
Fig. 1. Lipids regulate exocytosis by different mechanisms. This figure presents different ways used by lipids to regulate vesicle fusion during exocytosis. Lipids, such as lysophosphatidic acid present a conic shape that facilitate membrane curvature during exocytosis influencing this process in consequence (represented in green). In addition lipids could be incorporated into the proteins constituting the molecular machinery to for example stabilize membrane attachments as it happens with palmitoylation of SNAP-25, synaptobrevin, synaptotagmin, CSP and Rab proteins (in blue in the figure). Other lipids such PIP2 could be acting as molecular beacons for the guidance of the vesicles to secretory active sites (in yellow). Finally, signalling lipids could interact directly with SNARE proteins and promote vesicle fusion. This is the case of AA influencing syntaxin 1 activity or sphingosine facilitating an open conformation of synaptobrevin II and favouring SNARE complex formation (in red in the figure).

Signalling lipids, new players in the regulation of exocytosis

In neuroendocrine cells SNAREs proteins are located in the fluid mosaic of the plasma membrane composed of a diversity of lipids including phospholipids, sphingolipids and cholesterol [49], and stabilized by a matrix of cytoskeletal elements forming the dynamic cytoarchitecture of active sites [50,51]. In this environment, the activity of phospholipases could release either saturated and also polyunsaturated fatty acids (PUFAs) that normally are present in the sn-2 position [52], and often, the released PUFAs could act as a intracellular messengers regulating a diversity of cellular processes including exocytosis [25,53–57]. Specifically, phospholipase type A2 (PLA2) acting in the sn-2 site release lysophospholipids and free unsaturated fatty acids, and the latter ones can diffuse into cytosol

where they interact with their targets of action, likely in hydrophobic domains [58,59]. Certainly, different elements of the molecular machinery of exocytosis are among these targets, since it has been demonstrated that inhibitors of PLA2 influence exocytosis in neuroendocrine cells [57,60,61], and addition of snake PLA2 neurotoxins alter secretion in neuronal [62–65] and chromaffin cells [66,67] *via* a variety of mechanisms. In the latter secretory model, it is quite illustrative that other phospholipases such as phospholipase C (PLC) and phospholipase D (PLD) have been implicated in catecholamine secretion [68–71], in this cases the activation of PKC by diacylglycerol mediates the PLC pathway [70,72], whereas the generation of phosphatidic acid seems to be associated with PLD signalling driving the enhancement of exocytosis [68,69,71,73]. In all these cases the generation of a signalling lipid has been demonstrated to be essential to

influence different elements of the secretory pathway. From now on we will focus on the action of these compounds over the molecular constituents of the exocytotic molecular machinery.

Signalling lipids interact directly with the fusion machinery

After the release of signalling lipids such AA or sphingosine from the lipid bilayer, these lipids could diffuse and interact with SNARE proteins and therefore regulate the activity of these fusogenic proteins. The first report of a direct interaction of signalling lipids with SNAREs was reported in 2005 when the direct administration of AA or the treatment with PLA2s was demonstrated to enhance the formation of the SNARE complex in synaptic membrane preparations [9]. One of the most remarkable characteristic of this potentiation is that AA could interact with syntaxin-1 even in the presence of Munc-18 which stabilizes a closed conformation of syntaxin-1 (Fig. 1) [8,9], this may suggest that this lipid could penetrate into the hydrophobic zones of syntaxin-1 without altering the native dimers of syntaxin-1/Munc-18. This may be a basic principle of AA activation of syntaxins since it was also reported to occur with the syntaxin-3 isoform [8].

The importance of this mechanism for the regulation of syntaxins was later stressed when we found that the protein α-synuclein, implicated in the phatogenesis of Parkinson's disease, was found to sequester AA preventing the enhancement of SNARE complex formation caused by this lipid [74], thus providing new insights into the alteration of neurotransmission by the pathogenic α-synuclein.

More recently, in screening the ability of a diversity of lipids in modulating the formation of SNARE complexes, we found that only sphingosine and some derivatives were able to activate synaptobrevin 2 to engage SNAP-25-syntaxin heterodimers acting in the interphase between vesicular lipids and synaptobrevin (Fig. 1) [10]. This effect was dose-dependent with a EC50 ∼ 10 μM and resulted in the enhancement of the exocytosis in neuronal and neuroendocrine cellular models. Furthermore, in neurons from synaptobrevin 2 knockout mice no modulation of exocytosis by sphingosine was observed, thus stressing the implication of this vesicular SNARE in mediating the action of sphingosine activating neurosecretion [10]. Analysis of sphingosine-related compounds revealed two critical features of sphingosine to promote SNARE complex formation and enhance exocytosis: the length of the carbon chain and the positive charge of sphingosine. Furthermore, L-sphingosine was as active as the

D-sphingosine suggesting that it may act by perturbing the local environment of synaptobrevin [10].

In order to demonstrate that the endogenous sphingosine production could mimic these results, the activity of external sphingomyelinases (SMase) and intracellular ceramidases releasing sphingosine into the cytosol in isolated nerve terminals [10], or cultured chromaffin cells [54,75] was tested on potentiation of exocytosis. The obtained results support this mechanism and further implicate synaptobrevin 2 since the treatment of the cells with Botulinum Neurotoxin type D, cleaving vesicular synaptobrevin, prevented the enhancement of neurosecretion due to the production of sphingosine and derivatives.

It is important to note, however, that Camoletto and co-workers [76] found that sphingosine may act on syntaxin-1 facilitating the engagement with Munc-18. Thus, this mechanism will decrease the number of docked vesicles and increase paired-pulse facilitation in neurons.

In conclusion, there is substantial evidence for a direct interaction of signalling lipids with a variety of SNAREs and further work is needed to establish the precise molecular mechanisms involved in such interactions associated with the regulation of the secretory activity of neuronal and neuroendocrine cells.

Signalling lipids increase the frequency and quantal release of neurotransmitters

How do signalling lipids affect the exocytotic process?. Well, if these lipid messengers potentiate the formation of SNARE complexes it is predicted that they will enhance secretion, and in the case of sphingosine, this has been demonstrated in melanotrophs, chromaffin cells, isolated nerve terminals and hippocampal neurons [10]. Since, exocytosis is a multistep process involving the translocation of vesicles to the plasma membrane, the 'priming' or maturation of the vesicles to be in a 'ready-releasable' state, and the final fusion of the membranes to release the vesicular content [15,17,77–79], it is important to define the different stages of this process altered by signalling lipids and this required the use of biophysical techniques with the capability of analysing fusion at the level of individual vesicles.

In 2013, two groups used such techniques to study the effect of sphingosine over the exocytosis in different cellular systems. Zorec's group from Ljubljana University applied the capacitance technique [80,81] to resolve unitary exocytotic events in pituitary lactrotrophs finding that sphingosine increases the frequency of the fusion of small vesicles and also larger

dense vesicles [82]. They also observed that sphingosine promoted the full fusion of large vesicles whereas smaller vesicles tent to fuse in the 'kiss and run' mode [83], only partially releasing their vesicular content, leading to the conclusion that the vesicle size was an important factor favouring the shift of fusion mode caused by sphingosine.

Simultaneously, our group at the Institute of Neurosciences of Alicante performed experiments in adrenomedullary chromaffin cells, using the amperometry technique [84] to detect the release of catecholamines from individual fusion events by their oxidation at the tip of a carbon fibre [54]. In our study, sphingosine and derivatives were produced by SMase treatment of the cells, and resulted in the increase of the amount of catecholamines released in individual fusions with detection of changes in the kinetics of the process suggesting changes in the mode of fusion of the vesicles. In addition, AA was also employed to show a 2–3 fold increase in the amount of catecholamines release per individual event, again implying that in the control situation and with chromaffin cells stimulated by depolarization, the release is suboptimal (kiss and run mode) and that signalling lipids promoted the full granular fusion.

Later on, the whole cell and on-cell capacitance techniques were used to study if sphingomyeline derivatives affect the release of different types of vesicles in chromaffin cells [75], and the results demonstrated an increase in the frequency of the release of small vesicles as well as large dense granules in agreement with the results obtained in lactotrophs.

In conclusion, the experiments performed with techniques allowing the high temporal resolution of secretory events in neuroendocrine cells demonstrated that signalling lipids increase the frequency of fusion of clear small vesicles as well as large dense granules and that these lipids are able to favour a change in the mode of exocytosis from the transient fusion pore opening characteristic of the 'kiss and run' mode to the full extent fusion collapsing the vesicle membrane.

FTY-720, an analogue of sphingosine revealed multiple possible targets for signalling lipids derivatives

The sphingolipid signalling pathway is important for the regulation of multiple physiological processes in the brain [85–89], including neurotransmission [52,90–92], and for the pathologies associated with neuronal disorders [88,93–95]. Therefore, molecules designed to mimic these compounds could interfere with the normal and pathological neuronal pathways and be useful as potential pharmacological tools. This is the case with

FTY-720 also known as Fingolimod, an analogue of sphingosine that has been used extensively as an immunosuppressant agent [96], and moreover, it has been recently approved for treatment of relapsing remitting multiple sclerosis [11,97]. FTY-720 crosses the blood brain barrier [98], and like sphingosine is phosphorylated, allowing it to interact with the receptors of sphingosine-1P mediating the egression of lymphocytes and causing immunosuppression (Fig. 2) [99].

Very recently it was demonstrated that FTY-720 readily imitates sphingosine in its ability to interact with synaptobrevin promoting SNARE complex formation and increasing exocytosis from neuronal and neuroendocrine cellular systems [14]. This drug at concentrations around 10–20 μM (very similar to the sphingosine range), was able to enhance the frequency of glutamate release from rat synaptosomes, secretion from melanotrophs and chromaffin cells, and the neurotransmission from cultured rat hippocampal neurons (Fig. 2). In addition, FTY-720, also shares with sphingosine the modulation of the mode of exocytotic fusion, as it augments the amount of neurotransmitter release per individual fusion event [14].

Nevertheless, FTY-720 could be having a complex action on secretion, as it has been found to inhibit the release of cargo from different types of vesicles in cultured rat astrocytes due to a decrease in vesicle mobility [100]. Both sphingosine and FTY-720, caused an impaired access of the vesicles to releasable sites, an effect that has been associated with an alteration of calcium dynamics by signalling lipids in this cellular system [101].

The results from astrocytes are in apparent contradiction with those reported in neuroendocrine systems, nevertheless, we have found recently that in chromaffin cells FTY-720 has a dual effect on catecholamine release. In this neuroendocrine model, incubation with FTY-720, increases the frequency of vesicle fusions during the first round of cell stimulation by depolarization but decreases the amount of vesicles recruited in subsequent stimulations, (V. Garcia-Martinez, J. Villanueva, Y. Gimenez-Molina & L. M. Gutiérrez, unpublished results). Furthermore, by using FRET, we have observed that FTY-720 interacts at the molecular level with SNARE clusters in chromaffin cells as was previously described for sphingosine and AA [54]. In addition, FTY720 could also interact with F-actin governing the motion of the vesicles in the close proximity of secretory sites [102,103]. Thus, FTY-720 seems to influence secretion through interaction with several cellular targets, and remarkably we have found recently by using electron microscopy that this compound also promotes the homotypic fusion of vesicles and the heterotypic fusion of vesicles and mitochondria in the
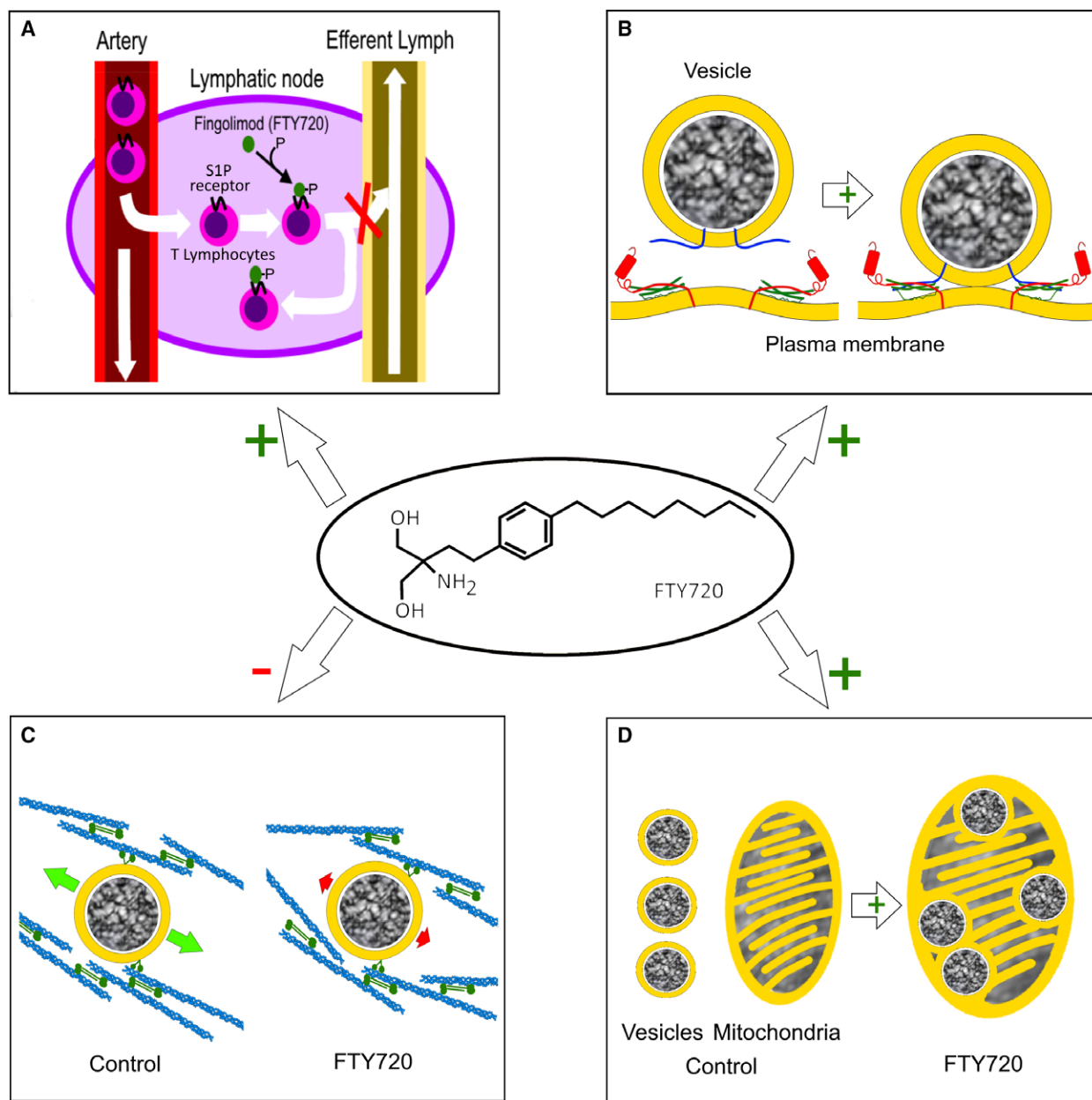
Fig. 2. Targets of the sphingomimetic drug FTY-720. The analogue of sphingosine FTY-720 was first characterized as an immunosuppressor drug when in its phosphorylated form binds to sphingosine 1-P receptors causing lymphocyte egress (A). This drug has been proved to mimic sphingosine activating synaptobrevin II and increasing the formation of the SNARE complex leading to the enhancement of neurosecretion (B). In addition, FTY-720 has been shown to inhibit the motion of the vesicles in astrocytes and chromaffin cells interacting with the F-actin cytoskeleton (C). Finally, and very recently, we have observed that FTY-720 could induce the homotypic fusion of vesicles and the heterotypic fusion of mitochondria with vesicles in the chromaffin cell cytosol (D, mixed organelles product of vesicle-mitochondria fusion).

cytosol of chromaffin cells (Y. Gimenez-Molina, V. Garcia-Martinez, J. Villanueva, B. Davletov & L. M. Gutiérrez, unpublished results, Fig. 2), these intriguing findings may suggest that the characterization of the cellular targets of FTY-720 is an open research subject requiring further experimentation.

Conclusions and perspectives

In conclusion, our understanding of the role of lipids in the process of the release of neurotransmitters and hormones by the exocytotic fusion of the vesicular and plasma membranes has evolved drastically in the last

20 years from being the mere structural components of these membranes to a more active and direct function in modulation of the proteins constituting the molecular machinery of membrane fusion. Today, it is well-accepted that in addition to this structural role, certain lipids such lysophospholipids could be helping to adopt the membrane curvature favouring membrane fusion [24,25], lipids such PIP2 are produced and transported to the active sites to act as a molecular beacons attracting vesicle movement towards these specific sites for preferential fusion [30], and proteins are modified by addition of palmitate chains to support membrane anchoring and stabilization [37].

Moreover, lipids such AA and sphingosine, produced by the action of phospholipases and diffusing into the cytosol acting as signalling lipids have been shown to interact with SNARE proteins and activate the formation of the fusogenic SNARE complex therefore facilitating neurotransmission in neuronal and neuroendocrine cells [8–10]. Interestingly, these lipids modulate not only the frequency of vesicle fusion but also the fusion pore behaviour promoting the full fusion mode over the partial release by the 'kiss and run' mode [54,82], thereby suggesting that these signalling lipids could be fine-tuning the amount of neurotransmitter release per secretory event to adapt to specific functional requirements.

The importance of these signalling lipids was stressed when FTY-720 (Fingolimod), a structural analogue of sphingosine was approved as the first drug for oral treatment of relapsing multiple sclerosis [11]. This therapeutic use is based on the immunosupression properties of the phosphorylated form of FTY-720 binding to sphingosine 1-P receptors and supressing lymphocyte egress [96]. In recent years, FTY-720 has been found to affect a plethora of physiological processes including neuronal gene expression, axonal growth, and regeneration [104], suggesting that this drug may influence a variety of aspects of the physiology of neurons. Therefore, the finding that FTY-720 mimics the properties of signalling lipids towards the activation of SNAREs and the parallel potentiation of exocytosis was fundamental to understand the possible mechanisms underlying the role of FTY-720 in neuronal function.

The results obtained with FTY-720 may indicate that signalling lipids and related drugs could be acting on multiple targets involved in different cellular and pathological processes explaining why this compound stimulates neuronal function and regeneration [104], and benefits neuroprotection in murine disease models [105], ischaemia [106,107], excitotoxicity [108], and can even improve the recovery of memory and learning in neurological disorders [109–112].

In summary, the study of the molecular mechanisms associated with the physiological regulation of neurosecretion by signalling lipids promises not only the understanding of basic mechanisms governing the secretory activity in neuronal and neuroendocrine cells but also the possible design of new therapeutic agents against neurological disorders.

Acknowledgements

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