FEBS Letters

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REVIEW ARTICLE

Phases of the exocytotic fusion pore

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(Received 9 July 2018, revised 25 August 2018, accepted 28 August 2018, available online 1 October 2018)

doi:10.1002/1873-3468.13234

Edited by Wilhelm Just

Membrane fusion and fission are fundamental processes in living organisms. Membrane fusion occurs through the formation of a fusion pore, which is the structure that connects two lipid membranes during their fusion. Fusion pores can form spontaneously, but cells endow themselves with a set of proteins that make the process of fusion faster and regulatable. The fusion pore starts with a narrow diameter and dilates relatively slowly; it may fluctuate in size or can even close completely, producing a transient vesicle fusion (kiss-and-run), or can finally expand abruptly to release all vesicle contents. A set of proteins control the formation, dilation, and eventual closure of the fusion pore and, therefore, the velocity at which the contents of secretory vesicles are released to the extracellular medium. Thus, the regulation of fusion pore expansion or closure is key to regulate the release of neurotransmitters and hormones. Here, we review the phases of the fusion pore and discuss the implications in the modes of exocytosis.

Keywords: amperometry; capacitance; exocytosis; fusion pore; kiss-and-run; patch clamp

A crucial event in evolution was the generation of individual organisms with a lipid bilayer to separate them from the environment. Unicellular organisms could then control their internal milieu and perform vital functions, avoiding external fluctuations. The cell membrane, at the same time, allows capturing nutrients and expelling debris, maintaining isolation simultaneously. Ion channels and transporter proteins fulfilled the exchange of ions or small molecules in and out of the cell. The fusion or fission of membranes was the solution for exchanging bigger molecules and the renewal of membrane components. Fusion and fission of membranes are not only important for the exchange of substances with the outside but is also the vehicle to transfer substances in different intracellular compartments and the nucleus. In addition, fusion and fission occur maintaining the cell interior isolated from the outside.

Understanding membrane fusion or fission is not trivial. Perhaps, a simple example can provide clues, for the nonexpert in the field, to unravel the underlying phenomena behind this important cellular function, such as making soap bubbles [1]. One can get bubbles when applying a slight air pressure through a thick lipid layer sitting in a plastic ring. As air passes through the lipid, thinner and thinner lipid layers develop until getting a simple lipid bilayer. Bubble size is inversely proportional to the surface tension generated by the lipid bilayers (a trick to form enormous bubbles are to add glycerin or sodium bicarbonate to the soap water). Sometimes, a smaller bubble forms inside a bigger one and, with time, the small bubble fuses with the largest without bursting. In addition, in the process of generating a large bubble, smaller ones can excise in a process similar to what happens during the fission of membranes inside cells. Fusion and

Abbreviations
CgA, chromogranin A; CgB, chromogranin B; Cgs, chromogranins; LDCV, large dense core vesicles; SLV, synaptic-like vesicles; Syt-1, synaptotagmin 1; Syt-7, synaptotagmin 7.

fission are intrinsic properties of lipid bilayers, endowing intracellular life with a simple mechanism of isolation and trafficking.

Although the fusogenic property of lipid bilayers is obvious, as noted in the example described before, it is now clear that what makes two phospholipid bilayers to fuse spontaneously is their high membrane tension and curvature [2]. These forces are sufficient to generate the fusion of membranes without the need of previous dehydration or elimination of charges on the surface of the bilayer [2,3]. Numerous models have been proposed of how the fusion intermediate stages are formed (hemifusion, stalk) [3–5] and progress to a dynamic fusion pore. The fusion pore can be stabilized [6] by peptides or a number of proteins related to the SNARE complex [7] or the cytoskeleton (see for review [8,9]), and can be controlled under certain experimental conditions giving rise to a potential target for cellular regulation of hormone and neurotransmitter release.

Direct visualization of the fusion pore

The fusion pore is the molecular structure that transiently connects two lipid membrane. Once formed, the fusion pore either closes or expands until full merging of the fusing membranes. Early freeze fracture scanning electron microscopy studies revealed fusion pores in prokaryotes and eukaryotes, suggesting that membrane fusion and fusion pore formation are a highly preserved phenomenon in evolution. Outstanding images of early fusion pores were shown in the mucocyst discharge of Tetrahymena [10], as well as in invertebrates like amebocytes from Limulus [11]. In vertebrates, fusion pores have been captured in nonexcitable, like mast cells [12], and excitable cells, both in Torpedo [13] and mammalian cells like chromaffin cells [14] and the neuromuscular junction [15]. However, with conventional electron microscopy, pore diameter appears much larger (20–30 nm), and the structure seems to be purely lipidic. An interesting fact of the scanning electron microscopy and freeze fracture images is that no particles ('proteins') appear at the vesicular and plasma membrane, contacting zone, but at the sides of the fusion pore. This is a strong evidence that at the beginning the fusion pore is composed of pure lipids [16,17]. These techniques showed 'snap shots' of the fusion pore but not its dynamics. Only very recently it has been possible to see the dynamics of the fusion pore with super-resolution techniques (STED microscopy) in chromaffin cells of the adrenal medulla, with pores expansion between 0 and 490 nm within 26 ms to seconds [18]. One of the major predictions from all of these observations is that the fusion pore is initially lipidic.

The lifetime of the fusion pore

The first electrophysiological measurements of membrane fusion were the remarkable work of Kenneth Cole [19], studying fertilization of sea urchin [19] and arbacia eggs [20]. These experiments served to establish an electrical model of the fusion pore, that later became the theoretical basis of the well time-resolved impedance techniques applied in eukaryotic cells. In the last decades, fusion pore measurements have improved, in temporal resolution and sensitivity. From seconds, as in the sea urchin eggs [21], to milliseconds in beige mouse mast cells [22–24]. The recording of single fusion events in bovine chromaffin cells [25] and rat peritoneal mast cells [26] has revealed that at first the fusion pore forms and can expand in milliseconds, suffer erratic conductance fluctuations, and close again. Although erratic conductance fluctuations may occur during its opening, the regular behavior of fusion pore expansion is exponential. Taken together, these results suggest that a purely lipid structure is responsible for the expansion of the fusion pore, since there are no proteins known with such an irregular behavior.

The initial conductance of the fusion pore was determined for the first time in peritoneal mast cells of beige mouse, which has giant secretory granules. The first use of the beige mouse preparation was done by the group of Joshua Zimmerberg [24], where they demonstrated that fusion pore formation precedes swelling and release of secretory contents. In this preparation, the group of Wolfhard Almers managed to measure, in a brilliant experiment, the current produced by the discharge of the positive potential of the vesicular interior with respect to the extracellular space through the fusion pore. It was determined that the pore conductance was about 230 pS [22], which was a conductance very similar to the conductance of a gap junction channel, and suggested a proteinaceous nature of the fusion pore. Subsequent studies in horse eosinophils [27], neutrophils [28], and mast cells [29] showed that the conductance of the fusion pore could be as low as 50 pS, which suggested that the size of the fusion pore probably depends on the size of the vesicle under study. Moreover, in pancreatic beta cells, where large dense core vesicles (LDCV) and synaptic-like vesicles (SLV) coexist, the fusion pore conductance is of 203 ± 53 pS in LDCV and 54 ± 16 pS in SLV. This corresponds to pore diameters of 1.4 ± 0.1 in LDCV and 0.8 ± 0.1 in SLV, suggesting that the

initial pore size depends on the size of the secretory vesicle [30]. In addition, subnanometer fusion pores have also been observed with electrophysiological methods and complemented with optophysiological data in pituitary secretory cells [31]. The fusion pore can also be stable and stabilized by a number of circumstances and can last for seconds with a diameter of tens of nanometers [18]. The group of Julio Fernandez proposed that the fusion pore is lipidic, but held and modulated by a scaffold protein that couples stimulus with secretion [32]. For several years, the lipid or protein nature of the primitive fusion pore at exocytosis was postulated and debated between the groups of Almers [33] and Fernandez [32,34]. Although this issue is not completely solved nowadays, what seems clear is that once formed, the fusion pore expands by a mixture of proteins and lipids.

How many stages of the fusion pore exist?

Admittance experiments performed with patch clamp whole cell to determine the plasma membrane surface area together with the simultaneous measurement of the release of secretory products by amperometry have unequivocally shown the phases of the fusion pore during exocytosis (Figs 1 and 2). These experiments allowed measuring the size of the secretory vesicle with great precision, the quantity of serotonin or catecholamines molecules released to the extracellular space, and the concentration of secretory products inside the vesicle. Moreover, offline analysis of admittance allows the determination of fusion pore conductance as it first opens and its later dynamics in correlation with the amperometric signal (Figs 1 and 2). From these recordings, the fusion pore can be divided into three distinctive phases: (a) Initial formation, before any content exits the vesicle; (b) Slow expansion; and (c) full expansion and membranes merging. The fusion pore opens with a conductance of 20–50 pS, grows with a nonwell-defined expansion kinetics without substates of conductance, and finally expands to a size, such as that observed in electron microscopy studies, equivalent to the diameter of the secretory vesicle. In the last few years there is an increasing interest to determine how and how many proteins (v and t-SNAREs) are required to form the pore [35–37]. Synaptotagmin-1 might also play a significant role in the early phase of fusion pore formation, since several of them together can spontaneously form ring-like structures that may participate at this stage [38], and even promote fusion in a Ca^{2+}-dependent manner *via* the binding of Ca^{2+} ions and phospholipids to the C2B domain [39]. Protein insertion into the membrane is important to energize and trigger fast fusion, as it occurs in regulated exocytosis. Once formed, the time taken for the fusion pore to expand depends critically on the vesicle radius. For example, the mean open time of secretory vesicles from beige mouse mast cells, which have a mean diameter of 2–4 μm, is on average 1 s, and its distribution is exponential [30]. In rat peritoneal mast cells, where granules are smaller (circa 1 μm), the size is about 100 ms [40], in chromaffin cells, about 10 ms [41], in cells from glomic cells of the carotid body, about 3.5 ms [42], and neurons from the central nervous system of the leech 1.3 ms [43]. During the slow expansion of the pore, there is an observable amperometric foot. These values, except from the leech and carotid body, came from the combination of patch clamp whole-cell admittance and the independent determination of secretory products by amperometry, where the distinctive phases of the pore are well established. In all secretory systems, the secretory products detected are positively charged molecules (noradrenaline, adrenaline, serotonin, histamine), which are trapped within a proteinaceous matrix [44]. The amount of secretory products released during this phase of expansion of the fusion pore is small, compared to the amount stored inside the vesicle and produces the amperometric foot, which was first observed in bovine chromaffin cells [45–46]. In mast cells, the mean charge of the amperometric foot represents between 1% and 2% of the vesicular contents and its time course is linearly correlated with the conductance of the fusion pore [40,47] in agreement with that found in chromaffin cells [48]. Apparently, during the slow expansion of the fusion pore, only a small amount of the total intragranular serotonin leaks out [40]. During this slow phase, the pore can fluctuate in size, and in some cases close completely giving rise to a transient fusion ('kiss-and-run') event with partial release. The amount released during 'kiss-and-run' depends on the size of the vesicle, the binding properties to the proteinaceous intragranular matrix, and the nature of the transmitter substance [49,50]. The term 'kiss-and-run' was proposed by Fesce *et al.* [51], after the observation of admittance and amperometric recordings which revived an old debate on vesicle fusion at the neuromuscular junction between the work of Heuser and Reese [52] in the USA and Cecarelli [53] in Italy. On occasions, long duration events leads to the complete emptying of the vesicle [40], these events are rare [54].

The trapping of secretory products within the vesicle has two important consequences for release. During the foot, the amount of molecules released from large

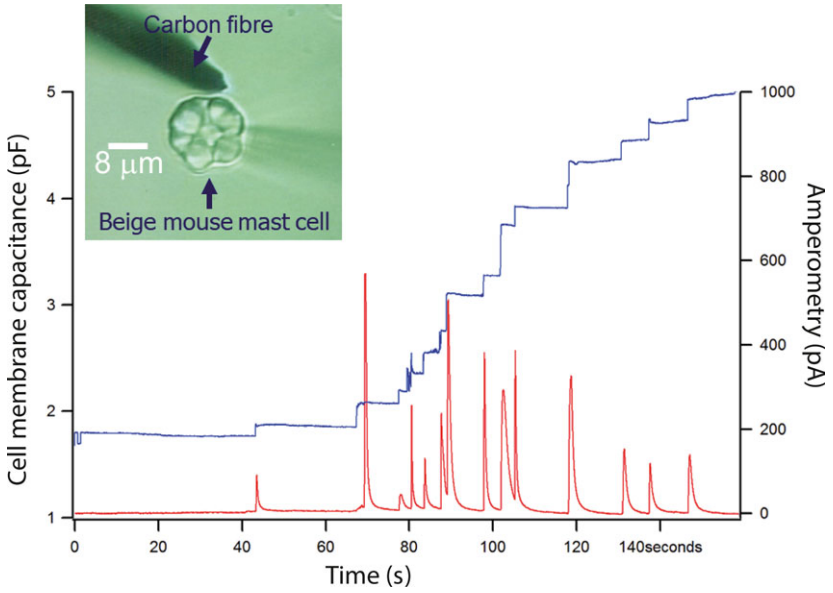

Figure 1. Simultaneous recording of whole-cell patch clamp membrane capacitance (blue) and amperometry (red) in a beige mouse mast cell. Exocytosis is triggered by cytosolic dialysis with GTPγS [25]. The beige mouse has been used classically to characterize the fine details of single vesicle exocytosis [19–21,70]. It served to unambiguously determine that exocytosis promotes an increase in cell surface area. The release of serotonin (and histamine, depending on the holding potential) can be detected by placing a carbon electrochemical detector over the cell surface (picture). Each step increase in cell membrane capacitance coincides, with certain and variable delay, with an amperometric spike. This type of recording demonstrated that secretory vesicle fusion release transmitters. Note the correlation between the step size in capacitance and the height of the amperometric spike. Notice also that every step is accompanied by a spike, regardless of the location of vesicle fusion over the cell membrane.

vesicles is relatively low in comparison with their total content, while for chromaffin vesicles or the central nervous system is much larger. In central nervous system synapses, almost total release can occur in 250 μs [50]. Figure 2C compares a real amperometric recording from a beige mouse mast cell granule (left) with one simulated assuming the that all serotonin molecules inside the vesicle are in solution (10 mM, calculated from the integral of the amperometric spike and the size of the vesicle with whole-cell patch clamp). In the second case, the pore conductance and the granule size were equivalent to the true recording. As it can be observed, in the simulation, the foot is much larger and the amperometric spike much smaller than in the real case. From this comparison it can be implied that the length of the amperometric foot is the only distinctive property about the fusion pore, since its amplitude not only depends on the size of the fusion pore but the on-and-off kinetics of the neurotransmitter to the intragranular matrix.

One important problem to consider is to define what is and what is not a fusion pore and what are the experimental approaches that one can use for measuring its properties and regulation. Cell-attached capacitance is a very reliable method to study the formation and biophysical properties of fusion pores, even more when it is combined with amperometry in the same pipette (patch amperometry). However, technically this method is very difficult to implement and to date only few laboratories have been successful with it. The study of prespike features observed by amperometry—the so-called 'foot'— offers an alternative. However, foot data should be taken with caution, as carbon fiber electrodes cannot distinguish whether a given foot is coming from the spike or from another adjacent release. Also, it should be noted that the initial points of a foot are difficult to set without capacitance correlation, as foot current accounts of a few pA over the basal noise. To carry out foot measurements it becomes crucial to perform blind analysis to prevent evaluator biases [55] but if this caution is taken it is seldom indicated. For this reason, one has to be cautious when making conclusions solely based on amperometry recordings.

Although the analysis of the ascending part of amperometric spikes cannot measure the diameter of a given fusion pore, its slope would be correlated with its expansion kinetics [48].

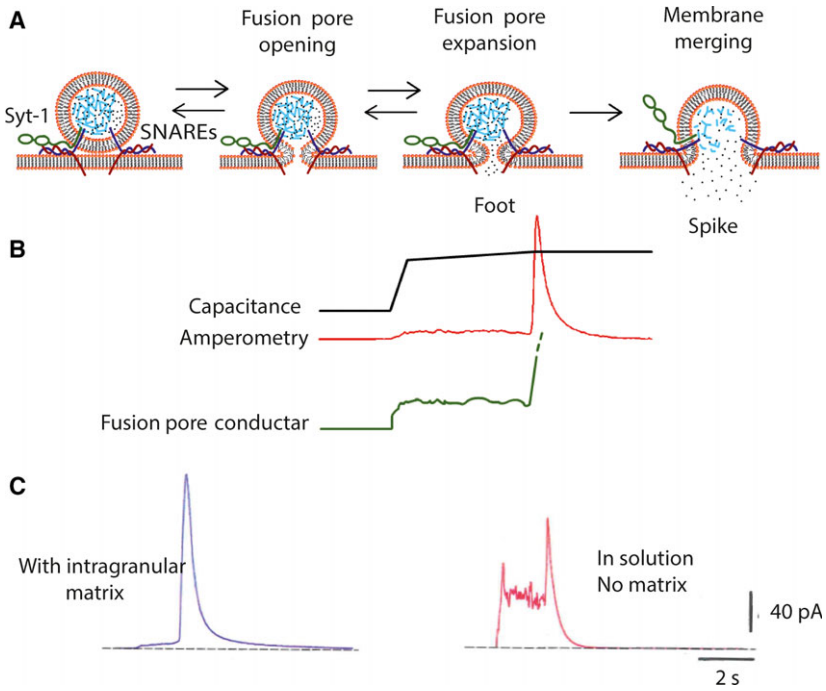

Figure 2. (A) Sequential model of vesicle fusion. Proteins of the fusion complex are simplified for clarity. Only t- and v-SNAREs and synaptotagmin are sketched. A vesicle passes through; (a) initial fusion pore formation with no release of cargo, (b) fusion pore expansion with release of a small amount of cargo probably in solution within the vesicle, giving rise to an amperometric foot. The final dilation of the pore leads to a full merging of vesicle and plasma membranes. (B) Fusion pore conductance during this phase parallels the amperometric signal. This applies for mast cells granules. In chromaffin granules and smaller granules, the amperometric feet can decrease in amplitude. (C) A real recording as depicted above (with intragranular matrix) (left), and a simulated foot and spike with the assumption that all serotonin contents are in solution (right). See the large amplitude foot that decreases in amplitude as the concentration inside the vesicle decreases. Notice the small and fast amperometric spike (adapted from [80]).

Fusion pore regulation in chromaffin granules

The first reported regulation of the fusion was on the acceleration of fusion pore expansion by intracellular Ca^{2+} in rat peritoneal mast cells [29] and horse eosinophils [27]. In bovine chromaffin cells, it is has been shown that increasing intracellular Ca^{2+} facilitates fusion pore opening, shifting the mode of exocytosis from 'kiss-and-run' to full fusion [56,57]. In rat chromaffin cells, the increase in external Ca^{2+} (> 20 mM) accelerates fusion pore expansion, but induces fusion pore reclosure [41]. During these events 'kiss-and-run' appears as a rapid expansion followed by a rapid closure of the pore without a full incorporation of the vesicular membrane into the plasma membrane.

The simultaneous recording of cell-attached patch clamp and amperometry (patch amperometry) [54] allowed the study of secretion in knockout and knock-in mouse of synaptotagmin-7 (Syt-7), and its Ca^{2+} dependence [58]. It has been demonstrated that the C2B domain of Syt-7 results essential for the full expansion of the fusion pore. The mutation of negatively charged residues that binds Ca^{2+} on the C2B promotes the closure of the fusion pore, giving rise to 'kiss-and-run' events. Moreover, in the absence of both C2 domains, A and B, as in the Syt-7 knockout mice chromaffin cells there is an increase in the lifetime of fusion pore expansion. Mutations of myosin II also increase fusion pore expansion [59].

There have been many studies about fusion pore kinetics-based solely on amperometry, either measuring foot characteristics or the properties of the amperometric spike. These results, although significant regarding the molecules regulating the fusion pore, must be taken with caution for the reasons expressed above. The characteristics and binding properties of the secretory products to the intragranular matrix might affect the characteristics of the amperometric foot. Ca^{2+} binding to Syt-1 probably shortens the amperometric foot [60], meanwhile the interaction of Ca^{2+} and Syt-1 is stronger when bound to phosphatidylserine [61]. Mutations of different residues of synaptotagmin or lengthening of the Syt molecule may

retard or shorten the amperometric foot [62,63]. We will not deal in detail here with all this set of experimental data, for a review see [64].

The role of vesicular matrix in the kinetics of fusion pore

Large dense core vesicles including chromaffin granules were described based in electron microscopy observations. They are characterized by electron-dense core, probably constituted by the aggregate of proteins and other soluble species thus forming the so-called vesicular matrix. Although our knowledge of the precise physico-chemical interaction of these components is still incomplete, there are cumulative evidence that matrix permit the accumulation of a large concentration of neurotransmitters and participates in the control of the exocytotic process. This also includes the dynamic of fusion pore. The role of the vesicular matrix in secretion of single secretory granules was first reported in beige mouse mast cells [24] and the theory behind the accumulation of transmitters inside secretory granules has been extensively studied [65,66].

Chromogranins (Cgs) are, by far, the most abundant intravesicular protein. They belong to a family of acidic proteins that are the source of several peptides with potent biological activity and also they are key actors in the process of vesicle sorting [67]. Nevertheless, the first role assigned to Cgs was to contribute to the accumulation of catecholamines in chromaffin cells [68]. The main Cgs found in chromaffin cells are chromogranin A (CgA), chromogranin B (CgB) and, to a lesser extent, secretogranin II (formerly called chromogranin C).

Since the discovery that most of the exocytosis of LDCV are partial (kiss-and-run, kiss-and-stay) and that the release of transmitters will be limited by the size of fusion pore, it became evident that a large matrix will not pass through it. As most of the soluble cargo species of LDCV are associated with matrix, it is feasible that the slow dissociation from the matrix together with the kinetics of expansion of fusion pore will govern exocytosis.

We started checking the role of Cgs on exocytosis the moment the first animals lacking Cgs became available [69–71]. Chromaffin granules from Cgs-KO mice had the catecholamine content reduced. However, there were differences depending on the KO strains. Hence, CgA-KO animals overexpressed CgB both facts induced a loss in catecholamines of ~ 34% [72]. Chromaffin cells from the CgB-KO mouse (which also overexpressed CgA) have a reduction in amine cargo of ~ 31% [71]. When both Cgs A &B were absent no overexpression of other granins were observed and the amine quantum size dropped ~ 42%. In spite of the reduction in net amine content, the lack of CgA accelerated exocytosis, whereas when CgB or both were absent, the process was slower. Two main phenomena might be behind these kinetic differences, (a) the dissociation from the matrix or (b) the dynamic changes of the fusion pore expansion; both are governing exocytosis.

Direct observations on fusion pore from CgB- or CgA&B-KO mice are not available. However, patch amperometry experiments performed on chromaffin cells from CgA-KO mice show that prespike phenomenon duration lasted a 52% more than its WT counterpart. However, dilation kinetics was similar [72], this is compatible with the idea that CgA contributes to the swelling of granules and its lack, regardless of the overexpression of CgB, keeps the fusion pore stable longer.

When CgA was overexpressed, in PC12 cells, there was an enormous increase in the quantum size. This was accompanied by an acceleration of fusion pore expansion as indicated by the increase in the ascending slope of amperometric spikes [73]. Using TIRFM and CgA coupled with EGFP to tag PC12 granule matrices, these authors showed that most of exocytosis (93%) occurred as partial phenomena, having a stable fusion pore lasting over 10 s [73].

The possible role of other intravesicular species

There is compiled evidence that changes in the vesicular composition will also affect the kinetics of fusion pore. As the major soluble component concentration are strongly dependent on pH gradient, the effect of its disruption indicates that catecholamines [74], Ca^{2+} [75], and probably ATP were drastically reduced after the application of bafilomycin 1A (a V-ATPase blocker) or by cell incubation with NH_4. Again, no direct data using cell-attached capacitance are available. However, these treatments resulted in a drastic reduction in the ascending slopes of amperometric spikes because of a reduction in quantum size.

The descriptions published about the role of calcium on fusion pore relays on the side of secretory vesicle where it is applied. Inside the vesicle, Ca^{2+} is a potent stabilizer of the fusion pore. Alés *et al.* [41] showed that elevation of extracellular Ca^{2+} present inside the pipette of patch amperometry switched the mode of exocytosis toward a reversible fusion with complete release of the granular contents. It is probable that part of this effect occurs by preventing the matrix

swelling. Conversely, when Ca^{2+} is increased in the cytosol or applying strong stimuli to chromaffin cells exocytosis mode moved to full fusion [56]. Secretory vesicles are no longer considered as a passive Ca^{2+} sink. Being by far the most important intracellular reservoir of Ca^{2+} its dynamic changes and its possible contribution to its own exocytosis is currently on debate.

ATP is, after catecholamines, the most abundant species in secretory vesicles from chromaffin cells, this is probably also true for most secretory cells [76]. When vesicular content of ATP was reduced by interfering the VNUT (the vesicular ATP carrier), the resulting total vesicular content of catecholamines was also reduced [77]. However, this quantum reduction was not accompanied with significant alterations of the ascending slopes of secretory spikes indicating that not all vesicular components of SV share the same function in the regulation of fusion pore kinetics [78].

Concluding remarks

Although initially the fusion pore was described just as an 'interesting phenomenon', cumulative experimental data have demonstrated its crucial role in exocytosis and in the regulation of partial and full release of secretory products. Its biological importance is reflected by the presence of proteins directly governing its formation, endurance, and closure. Even more, fusion pore allows the discrimination of releasable substances by altering its diameter or its opening/closure time during partial exocytosis. The fusion pore is regulated and can be stabilized, representing a controllable channel in kinetics and diameter [79]. This has important implications in the origin of disease, thus making the fusion pore a potential candidate for therapeutics.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Economy and Competitiveness (MINECO/FEDER BFU2017-82618-P) to RB and (MINECO/FEDER BFU2015-64536-R) to GAT. GAT was also supported by the Junta de Andalucia (BIO-209).

References

1 Isenberg C (1992) The Science of Soap Films and Soap Bubbles. Courier Corporation, Dover Publications, Inc., Mineola, NY.
2 Helm CA, Israelachvili JN and McGuiggan PM (1992) Role of hydrophobic forces in bilayer adhesion and fusion. *Biochemistry (Mosc.)* **31**, 1794–1805.
3 Helm CA, Israelachvili JN and McGuiggan PM (1989) Molecular mechanisms and forces involved in the adhesion and fusion of amphiphilic bilayers. *Science* **246**, 919–922.
4 Chernomordik LV, Melikyan GB and Chizmadzhev YA (1987) Biomembrane fusion: a new concept derived from model studies using two interacting planar lipid bilayers. *Biochim Biophys Acta* **906**, 309–352.
5 Chernomordik LV, Zimmerberg J and Kozlov MM (2006) Membranes of the world unite! *J Cell Biol* **175**, 201–207.
6 Jorgacevski J, Fosnaric M, Vardjan N, Stenovec M, Potokar M, Kreft M, Kralj-Iglic V, Iglic A and Zorec R (2010) Fusion pore stability of peptidergic vesicles. *Mol Membr Biol* **27**, 65–80.
7 Jorgacevski J, Potokar M, Grilc S, Kreft M, Liu W, Barclay JW, Bückers J, Medda R, Hell SW, Parpura V *et al.* (2011) Munc18-1 tuning of vesicle merger and fusion pore properties. *J Neurosci* **31**, 9055–9066.
8 Sharma S and Lindau M (2018) The fusion pore, 60 years after the first cartoon. *FEBS Lett*.
9 Hastoy B, Clark A, Rorsman P and Lang J (2017) Fusion pore in exocytosis: more than an exit gate? A β-cell perspective *Cell Calcium* **68**, 45–61.
10 Satir B, Schooley C and Satir P (1973) Membrane fusion in a model system. Mucocyst secretion in Tetrahymena. *J Cell Biol* **56**, 153–176.
11 Ornberg RL and Reese TS (1981) Beginning of exocytosis captured by rapid-freezing of Limulus amebocytes. *J Cell Biol* **90**, 40–54.
12 Chandler DE and Heuser JE (1980) Arrest of membrane fusion events in mast cells by quick-freezing. *J Cell Biol* **86**, 666–674.
13 Nickel E and Potter LT (1971) Synaptic vesicles in freeze-etched electric tissue of torpedo. *Philos Trans R Soc B Biol Sci* **261**, 383–385.
14 Schmidt W, Patzak A, Lingg G, Winkler H and Plattner H (1983) Membrane events in adrenal chromaffin cells during exocytosis: a freeze-etching analysis after rapid cryofixation. *Eur J Cell Biol* **32**, 31–37.
15 Heuser JE and Reese TS (1981) Structural changes after transmitter release at the frog neuromuscular junction. *J Cell Biol* **88**, 564–580.
16 Chi EY, Lagunoff D and Koehler JK (1975) Electron microscopy of freeze-fractured rat peritoneal mast cells. *J Ultrastruct Res* **51**, 46–54.
17 Lawson D, Raff MC, Gomperts B, Fewtrell C and Gilula NB (1977) Molecular events during membrane fusion. A study of exocytosis in rat peritoneal mast cells. *J Cell Biol* **72**, 242–259.
18 Shin W, Ge L, Arpino G, Villarreal SA, Hamid E, Liu H, Zhao WD, Wen PJ, Chiang HC and Wu LG (2018) Visualization of membrane pore in live cells reveals a dynamic-pore theory governing fusion and endocytosis. *Cell* **173**, 934–945. e12.

19 Cole KS (1968) Membranes, Ions and Impulses. University of California Press, Berkeley, CA.
20 Cole KS and Cole RH (1936) Electric impedance of arbacia eggs. *J Gen Physiol* **19**, 625–632.
21 Jaffe LA, Hagiwara S and Kado RT (1978) The time course of cortical vesicle fusion in sea urchin eggs observed as membrane capacitance changes. *Dev Biol* **67**, 243–248.
22 Breckenridge LJ and Almers W (1987) Currents through the fusion pore that forms during exocytosis of a secretory vesicle. *Nature* **328**, 814–817.
23 Alvarez de Toledo G and Fernandez JM (1988) The events leading to secretory granule fusion. *Soc Gen Physiol Ser* **43**, 333–344.
24 Zimmerberg J, Curran M, Cohen FS and Brodwick M (1987) Simultaneous electrical and optical measurements show that membrane fusion precedes secretory granule swelling during exocytosis of beige mouse mast cells. *Proc Natl Acad Sci* **84**, 1585–1589.
25 Neher E and Marty A (1982) Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proc Natl Acad Sci USA* **79**, 6712–6716.
26 Fernandez JM, Neher E and Gomperts BD (1984) Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature* **312**, 453–455.
27 Hartmann J and Lindau M (1995) A novel Ca2 + - dependent step in exocytosis subsequent to vesicle fusion. *FEBS Lett* **363**, 217–220.
28 Lollike K, Borregaard N and Lindau M (1995) The exocytotic fusion pore of small granules has a conductance similar to an ion channel. *J Cell Biol* **129**, 99–104.
29 Fernández-Chacón R and de Toledo GA (1995) Cytosolic calcium facilitates release of secretory products after exocytotic vesicle fusion. *FEBS Lett* **363**, 221–225.
30 MacDonald PE, Braun M, Galvanovskis J and Rorsman P Release of small transmitters through kiss-and-run fusion pores in rat pancreatic beta cells. *Cell Metab* **4**, 283–290.
31 Vardjan N, Stenovec M, Jorgačevski J, Kreft M and Zorec R (2007) Subnanometer fusion pores in spontaneous exocytosis of peptidergic vesicles. *J Neurosci* **27**, 4737–4746.
32 Monck JR and Fernandez JM (1996) The fusion pore and mechanisms of biological membrane fusion. *Curr Opin Cell Biol* **8**, 524–533.
33 Lindau M and Almers W (1995) Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion. *Curr Opin Cell Biol* **7**, 509–517.
34 Monck JR and Fernandez JM (1992) The exocytotic fusion pore. *J Cell Biol* **119**, 1395–1404.
35 Bao H, Goldschen-Ohm M, Jeggle P, Chanda B, Edwardson JM and Chapman ER (2016) Exocytotic fusion pores are composed of both lipids and proteins. *Nat Struct Mol Biol* **23**, 67–73.
36 Sharma S and Lindau M (2016) The mystery of the fusion pore. *Nat Struct Mol Biol* **23**, 5–6.
37 Wu Z, Thiyagarajan S, O'Shaughnessy B and Karatekin E (2017) Regulation of exocytotic fusion pores by SNARE protein transmembrane domains. *Front Mol Neurosci* **10**, 315.
38 Zanetti MN, Bello OD, Wang J, Coleman J, Cai Y, Sindelar CV, Rothman JE and Krishnakumar SS (2016) Ring-like oligomers of synaptotagmins and related C2 domain proteins. *ELife* **5**, pii: e17262.
39 Wang J, Li F, Bello OD, Sindelar CV, Pincet F, Krishnakumar SS and Rothman JE (2017) Circular oligomerization is an intrinsic property of synaptotagmin. *ELife* **6**, pii: e27441.
40 Alvarez de Toledo GA, Fernández-Chacón R and Fernández JM (1993) Release of secretory products during transient vesicle fusion. *Nature* **363**, 554–558.
41 Alés E, Tabares L, Poyato JM, Valero V, Lindau M and de Toledo GA (1999) High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism. *Nat Cell Biol* **1**, 40–44.
42 Ureña J, Fernández-Chacón R, Benot AR, Alvarez de Toledo GA and López-Barneo J (1994) Hypoxia induces voltage-dependent Ca2 + entry and quantal dopamine secretion in carotid body glomus cells. *Proc Natl Acad Sci USA* **91**, 10208–10211.
43 Bruns D and Jahn R (1995) Real-time measurement of transmitter release from single synaptic vesicles. *Nature* **377**, 62–65.
44 Fernandez JM, Villalón M and Verdugo P (1991) Reversible condensation of mast cell secretory products *in vitro*. *Biophys J* **59**, 1022–1027.
45 Chow RH, von Rüden L and Neher E (1992) Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells. *Nature* **356**, 60–63.
46 Wightman RM, Schroeder TJ, Finnegan JM, Ciolkowski EL and Pihel K (1995) Time course of release of catecholamines from individual vesicles during exocytosis at adrenal medullary cells. *Biophys J* **68**, 383–390.
47 Tabares L, Lindau M and Alvarez de Toledo G (2003) Relationship between fusion pore opening and release during mast cell exocytosis studied with patch amperometry. *Biochem Soc Trans* **31**, 837–841.
48 Schroeder TJ, Borges R, Finnegan JM, Pihel K, Amatore C and Wightman RM (1996) Temporally resolved, independent stages of individual exocytotic secretion events. *Biophys J* **70**, 1061–1068.

49 Katz B (1996) Neural transmitter release: from quantal secretion to exocytosis and beyond. *J Neurocytol* **25**, 677–686.

50 Neher E (1993) Cell physiology. Secretion without full fusion. *Nature* **363**, 497–498.

51 Fesce R, Grohovaz F, Valtorta F and Meldolesi J (1994) Neurotransmitter release: fusion or 'kiss-and-run'? *Trends Cell Biol* **4**, 1–4.

52 Heuser JE and Reese TS (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J Cell Biol* **57**, 315–344.

53 Ceccarelli B, Hurlbut WP and Mauro A (1973) Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J Cell Biol* **57**, 499–524.

54 Albillos A, Dernick G, Horstmann H, Almers W, de Toledo GA and Lindau M (1997) The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature* **389**, 509–512.

55 Machado DJ, Montesinos MS and Borges R (2008) Good practices in single-cell amperometry. *Methods Mol Biol Clifton NJ* **440**, 297–313.

56 Elhamdani A, Azizi F and Artalejo CR (2006) Double patch clamp reveals that transient fusion (kiss-and-run) is a major mechanism of secretion in calf adrenal chromaffin cells: high calcium shifts the mechanism from kiss-and-run to complete fusion. *J Neurosci Off J Soc Neurosci* **26**, 3030–3036.

57 Fulop T and Smith C (2006) Physiological stimulation regulates the exocytic mode through calcium activation of protein kinase C in mouse chromaffin cells. *Biochem J* **399**, 111–119.

58 Segovia M, Alés E, Montes MA, Bonifas I, Jemal I, Lindau M, Maximov A, Südhof TC and Alvarez de Toledo G (2010) Push-and-pull regulation of the fusion pore by synaptotagmin-7. *Proc Natl Acad Sci* **107**, 19032–19037.

59 Ñeco P, Fernández-Peruchena C, Navas S, Gutiérrez LM, de Toledo GÁ and Alés E (2008) Myosin II contributes to fusion pore expansion during exocytosis. *J Biol Chem* **283**, 10949–10957.

60 Wang C-T, Bai J, Chang PY, Chapman ER and Jackson MB (2006) Synaptotagmin–Ca^{2+} triggers two sequential steps in regulated exocytosis in rat PC12 cells: fusion pore opening and fusion pore dilation. *J Physiol* **570**, 295–307.

61 Zhang Z, Hui E, Chapman ER and Jackson MB (2009) Phosphatidylserine regulation of Ca^{2+}-triggered exocytosis and fusion pores in PC12 cells. *Mol Biol Cell* **20**, 5086–5095.

62 Bai J, Wang C-T, Richards DA, Jackson MB and Chapman ER (2004) Fusion pore dynamics are regulated by synaptotagmin t-SNARE interactions. *Neuron* **41**, 929–942.

63 Lynch KL, Gerona RRL, Kielar DM, Martens S, McMahon HT and Martin TFJ (2008) Synaptotagmin-1 utilizes membrane bending and SNARE binding to drive fusion pore expansion. *Mol Biol Cell* **19**, 5093–5103.

64 Chang C-W, Chiang C-W and Jackson MB (2017) Fusion pores and their control of neurotransmitter and hormone release. *J Gen Physiol* **149**, 301–322.

65 Uvnäs B and Aborg CH (1977) On the cation exchanger properties of rat mast cell granules and their storage of histamine. *Acta Physiol Scand* **100**, 309–314.

66 Marszalek PE, Farrel B, Verdugo P and Fernandez JM (1997) Kinetics of release of serotonin from isolated secretory granules. II. Ion exchange determines the diffusivity of serotonin. *Biophys J* **73**, 1169–1183.

67 Taupenot L, Harper KL and O'Connor DT (2003) The chromogranin-secretogranin family. *N Engl J Med* **348**, 1134–1149.

68 Blaschko H, Comline RS, Schneider FH, Silver M and Smith AD (1967) Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. *Nature* **215**, 58–59.

69 Mahapatra NR, O'Connor DT, Vaingankar SM, Hikim AP, Mahata M, Ray S, Staite E, Wu H, Gu Y, Dalton N *et al.* (2005) Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog. *J Clin Invest* **115**, 1942–1952.

70 Díaz-Vera J, Camacho M, Machado JD, Domínguez N, Montesinos MS, Hernández-Fernaud JR, Luján R and Borges R (2012) Chromogranins A and B are key proteins in amine accumulation, but the catecholamine secretory pathway is conserved without them, *FASEB. FASEB J Off Publ Fed Am Soc Exp Biol* **26**, 430–438.

71 Díaz-Vera J, Morales YG, Hernández-Fernaud JR, Camacho M, Montesinos MS, Calegari F, Huttner WB, Borges R and Machado JD (2010) Chromogranin B gene ablation reduces the catecholamine cargo and decelerates exocytosis in chromaffin secretory vesicles. *J Neurosci Off J Soc Neurosci* **30**, 950–957.

72 Montesinos MS, Machado JD, Camacho M, Diaz J, Morales YG, Alvarez de la Rosa D, Carmona E, Castañeyra A, Viveros OH, O'Connor DT *et al.* (2008) The crucial role of chromogranins in storage and exocytosis revealed using chromaffin cells from chromogranin A null mouse. *J Neurosci Off J Soc Neurosci* **28**, 3350–3358.

73 Dominguez N, Estevez-Herrera J, Borges R and Machado JD (2014) The interaction between chromogranin A and catecholamines governs exocytosis. *FASEB J Off Publ Fed Am Soc Exp Biol* **28**, 4657–4667.

74 Camacho M, Machado JD, Montesinos MS, Criado M and Borges R (2006) Intragranular pH rapidly modulates exocytosis in adrenal chromaffin cells. *J Neurochem* **96**, 324–334.

75 Camacho M, Machado JD, Alvarez J and Borges R (2008) Intravesicular calcium release mediates the motion and exocytosis of secretory organelles: a study with adrenal chromaffin cells. *J Biol Chem* **283**, 22383–22389.
76 Borges R (2013) The ATP or the natural history of neurotransmission. *Purinergic Signal* **9**, 5–6.
77 Estévez-Herrera J, Domínguez N, Pardo MR, González-Santana A, Westhead EW, Borges R and Machado JD (2016) ATP: the crucial component of secretory vesicles. *Proc Natl Acad Sci USA* **113**, E4098–E4106.
78 Estévez-Herrera J, González-Santana A, Baz-Dávila R, Machado JD and Borges R (2016) The intravesicular cocktail and its role in the regulation of exocytosis. *J Neurochem* **137**, 897–903.
79 Stenovec M, Kreft M, Poberaj I, Betz WJ and Zorec R (2004) Slow spontaneous secretion from single large dense-core vesicles monitored in neuroendocrine cells. *FASEB J* **18**, 1270–1272.
80 Fernández-Chacón R (1995) Estudio de la exocitosis mediante detección electroquímica y técnica de patch clamp en células cebadas. Ph.D. Thesis.