Biophysical Chemistry 126 (2007) 201–208

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

In search of the fusion pore of exocytosis

Meyer B. Jackson

Department of Physiology, University of Wisconsin, United States

Received 19 April 2006; accepted 11 May 2006
Available online 22 June 2006

Abstract

Research on calcium-triggered exocytosis has converged on the fusion pore as a critical kinetic intermediate. Using sensitive biophysical methods to record signals from living cells in the act of releasing neurotransmitter or hormone has provided clues about the structure and composition of fusion pores. The dynamics of fusion pore opening, closing, and dilating has revealed how specific proteins transduce a calcium binding signal to catalyze membrane fusion. The fusion pore determines how rapidly neurotransmitter is expelled from a vesicle into the synaptic cleft. This rate places constraints on the form of a synaptic response during different modes of release.

Keywords: Membrane fusion; Exocytosis; Synaptic transmission; SNARE proteins; Synaptotagmin; Neurosecretion

I worked in Julian Sturtevant's lab as a graduate student from 1973 to 1977. Gonul Velicelibi joined the lab at about the same time and we were Julian's last Ph.D. students. One particularly momentous change that took place while we were there was that we stopped calling him "Dr. Sturtevant" and began calling him "Julian" (at his suggestion, of course). I was a student in the Department of Molecular Biophysics and Biochemistry, and my interests were more biological than most of the people in the lab. The rigorous training I received from Julian in thermodynamics was of immense value. Although the subject of my research has changed dramatically over the years, thermodynamics and other fundamental subjects of physical biochemistry remain essential as I think about scientific problems. Julian set an especially good example to students with his hands-on approach to research. He never stopped doing his own experiments, and his practice is always with me as I continue to keep my own hands in the laboratory.

My research has turned to regulated exocytosis, which my lab studies using electrophysiological recording methods in living cells. Exocytosis is an essential process in neural and endocrine regulation whereby Ca^{2+} enters a cell and triggers the release of a chemical signal. The chemical to be released is stored in vesicles, and the release begins when the vesicle membrane forms a specialized contact with the plasma membrane. When this contact creates a continuous aqueous connection between the vesicle lumen and the extracellular fluid, we say that the contact has formed a fusion pore. Our efforts to elucidate the mechanism of Ca^{2+}-triggered exocytosis have focused on the fusion pore, using biophysical methods to probe its structure and dynamics. This article will summarize this work and discuss some of the implications for rapid synaptic transmission.

1. Background

The earliest efforts to characterize the fusion pore employed electron microscopy of rapidly frozen nerve terminals. These pictures revealed narrow fluid connections between the vesicle and the cell exterior with diameters as small as 20 nm [1]. However, in this study the authors pointed out that these stalks could have been narrower a few milliseconds earlier because the tissue froze after the fusion process was underway. The initial fusion pore might have grown before being frozen, thus eluding capture by the rapid freeze technique of Heuser and Reese. If one wants to know how the fusion pore materializes within the junction of the two membranes, this 20 nm pore tells us very little. The early effort of Heuser and Reese had more value in defining a goal than in providing insight into the structure of the earliest intermediates of membrane fusion.

Electrical recordings from living cells in the act of secretion ultimately gave more insight into the nature and properties of the initial fusion pore. Neher and Marty [2] measured the

E-mail address: mjackson@physiology.wisc.edu.

0301-4622/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2006.05.022

stepwise changes in capacitance (proportional to membrane area) that occurred in the adrenal chromaffin cell membrane when a single vesicle fuses. They observed that an upward step in capacitance is often immediately followed by a downward step of exactly the same size. This suggests that for a brief period of time (<1 s), the membrane of the vesicle is electrically coupled to the outside of the cell, but no mixing occurs between the two membranes. Since lipids are fluid and readily mix during membrane fusion, the reversible capacitance steps suggested that something unusual was going on. Either some form of scaffold holds the vesicle in place to prevent its collapse into the plasma membrane, or the molecular contact that creates an electrolytic pathway from the outside of the cell to the inside of the vesicle is not lipidic and blocks membrane mixing. The observation of these capacitance flickers has been repeated in other cell types containing very different types of vesicles [3,4]. It thus appears to be a general feature of biological exocytosis. Only when the contact lasts a long time and the fusion pore expands to a large size does membrane mixing begin to occur [5].

Further electrical measurements with a variety of techniques have provided estimates of the conductance of the initial fusion pore. For the smallest vesicles, such as those found at synapses, the conductance is only about 20 pS, and for larger dense-core vesicles the conductance is a few hundred pS [4,6]. These values are in the range found for ion channels and since the diameters of many ion channels can be estimated by size exclusion or by direct structural analysis, one can infer similar sizes for a fusion pore.

A crude approximate expression relates the dimensions of the fusion pore to the conductance. The pore is pictured as a cylindrical element of electrolyte with resistivity ρ, for which the macroscopic dependence of conductance (γ) on radius (a) and length (d) can be written as [7]

$$\gamma = \frac{\pi a^2}{\rho d} \tag{1}$$

This expression is only correct to within a factor of about 3 for the largest channels with diameters of the order of 1 nm, and its accuracy deteriorates rapidly as the diameter gets smaller [8]. This expression will be used below in an evaluation of how fusion pore properties can influence synaptic transmission.

Although the analogy between fusion pores and ion channels is a bit shaky, these kinds of arguments evoke a picture of a fusion pore as a gap junction formed by two connexin hemi-pores spanning and connecting two parallel adjacent lipid bilayers [6]. This model helps to formulate a clear and important question. If the fusion pore is formed by proteins spanning the two membranes, what are the identities of these proteins?

2. Fusion pores and molecular mechanisms of exocytosis

To address these and related questions, my laboratory developed an experimental program in which fusion pores could be studied in cells following molecular manipulations. We used PC12 cells, a rat pheochromocytoma cell line that releases catecholamines. These cells can be maintained in culture, and they will express proteins of interest when transfected with the appropriate DNA using electroporation.

PC12 cells release catecholamines such as norepinephrine, and this release can be monitored in real time from individual cells by carbon fiber amperometry. A carbon fiber electrode with a diameter of 5 μm is positioned under a microscope to touch the surface of a cell. The carbon fiber is polarized to 650 mV, a potential that exceeds the redox potential for norepinephrine. When a molecule of norepinephrine collides with the electrode surface it is oxidized to a quinone compound and releases two electrons. A typical dense-core vesicle of a PC12 cell contains about 50,000 molecules of norepinephrine, and the charge on the order of picocoulombs gives rise to a large and readily detected signal. Thus, this technique can be used to measure the release of a single vesicle. The experimental design is illustrated in Fig. 1A.

The single-vesicle release event looks like a spike, reflecting the rapid, nearly instantaneous expulsion of the entire content of the vesicle (Fig. 1B and C). These events contain an important feature that yields valuable information about the fusion pore. When one expands the scales of these records and looks

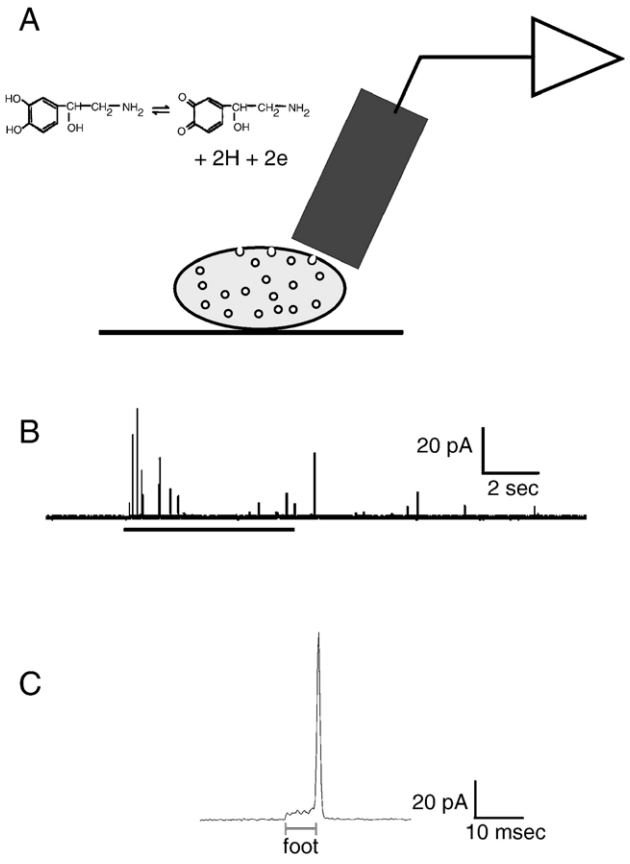

Fig. 1. Amperometric measurements of single-vesicle and single-fusion-pore release events. (A) A sketch of the carbon fiber electrode, recording current produced by the oxidation of released norepinephrine from a depolarized PC12 cell. The chemical conversion of norepinephrine is shown above. (B) A recording of several single-vesicle release events, triggered by depolarization of a PC12 cell with high-KCl (indicated by the bar below the trace). [41] (C) A single release event on an expanded scale shows the foot that signals an open fusion pore (data provided by Mr. Zhen Zhang).

carefully at the amperometric current that precedes the spike, one sees a "foot" caused by the slow release of a small amount of norepinephrine. This foot represents the flux of norepinephrine through the fusion pore. Fusion pore opening allows a small fraction of the vesicle's norepinephrine to escape during an early stage of exocytosis, before the vesicle membrane actually begins to fuse with the plasma membrane [9]. The amplitude of the foot current gives an indication of the fusion pore size and the duration tells us something about its stability (Fig. 1C). Studying each of these parameters provides insight into specific aspects of the molecular mechanism of exocytosis.

In our initial studies of fusion pores, we transfected PC12 cells with two different isoforms of synaptotagmin [10]. Synaptotagmin is a synaptic vesicle protein [11,12], that harbors two C2 domains with the capacity to bind Ca^{2+} and engage in interactions with a number of targets [13,14]. A large body of work implicates this protein as the Ca^{2+} sensor of exocytosis [15]. When Ca^{2+} enters a nerve terminal or neuroendocrine cell, it binds to synaptotagmin and in some way triggers vesicle fusion. We found that transfecting PC12 cells with synaptotagmin altered the duration of amperometric foot signals. PC12 cells express the main synaptotagmin isoform synaptotagmin I endogenously on their norepinephrine-containing dense-core vesicles [16]. Transfecting synaptotagmin I increases the levels of this protein and prolongs the open state of the fusion pore. Thus, the time interval from the appearance of the foot until its termination with the onset of the spike increases. Transfecting with another isoform, synaptotagmin IV, has the opposite effect, shortening the fusion pore lifetime [10]. Synaptotagmin IV is much less abundant and has no known Ca^{2+} binding activity. Yet it appears on dense-core vesicles when transfected into PC12 cells, and reduces the rate at which vesicles fuse in response to Ca^{2+}.

These effects of synaptotagmin on fusion pore lifetime were interpreted in terms of a simple kinetic model in which the open fusion pore can do two different things. The fusion pore can either close or it can dilate. If the rate constant for closure is α and the rate constant for dilation is β, then according to single-channel kinetic theory the mean lifetime of an open fusion pore is $1/(\alpha+\beta)$ [17,18]. The finding that altering the ratios of different synaptotagmin isoforms alters the mean fusion pore lifetime thus indicates that synaptotagmins exert some form of control over one or both of these two exit transitions of an open fusion pore. This places synaptotagmin within a fusion complex and suggests a role during a critical step in the evolution of the fusion pore. Subsequent work with mutant forms of synaptotagmin I suggested that Ca^{2+}-stimulated interactions with the SNARE proteins, syntaxin and SNAP-25 stabilize the open fusion pore [19]. Experiments with synaptotagmin I Ca^{2+} binding site mutations and with different Ca^{2+} concentrations suggested that Ca^{2+} binding to synaptotagmin I triggers the initial opening transition as well as a subsequent transition of an open fusion pore to a dilating state [20].

The experiments with synaptotagmin revealed actions on the fusion pore, but failed to address the question of the identity of the fusion pore's structural components. In an effort to answer this question we turned from fusion pore lifetimes to flux and used the amplitude of the foot current as an indicator of pore size. We reasoned that if a part of a protein lined the walls of the pore, then changes in these amino acids would alter the ease with which norepinephrine passed through the pore and thus change the amplitude of the foot signal.

We then needed to find candidate proteins that could contribute a domain to the fusion pore structure. We selected the t-SNARE protein syntaxin, because it is essential for exocytosis and it contains a segment that is thought to form a transmembrane α-helix. PC12 cells were transfected with a series of 16 mutants in which tryptophan replaced a native amino acid in the membrane anchor. Three mutants were found that reduced fusion pore flux [21], and these three residues fell along one face of a helical wheel (Fig. 2). This result thus identifies the membrane anchor of syntaxin as a structural component of the fusion pore. Electrical measurements of fusion pore conductance confirmed that these tryptophan mutants had narrower fusion pores. Further experiments with other mutations showed that increases in side chain volume at these sites produced a graded reduction in fusion pore flux. Finally, negative charge mutations increased the flux of

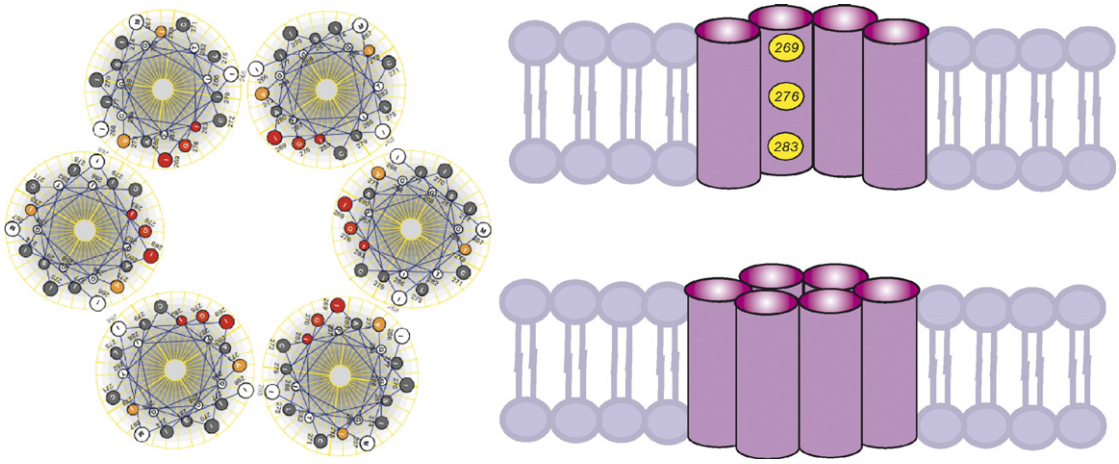

Fig. 2. Different views of a structural model of the part of the fusion pore formed by the membrane spanning segment of syntaxin. The helical segments from six molecules of syntaxin surround the fusion pore with the pore-lining residues (implicated by amperometry and conductance measurement) facing inward [41].

positively charged norepinephrine and positive charge mutations reduced the flux. Thus, there is an electrostatic interaction between these residues and the escaping neurotransmitter [22].

These results suggested that the half of the fusion pore through the plasma membrane is composed of the membrane anchor of syntaxin. The obvious protein to form the other half of the fusion pore through the vesicle membrane is the v-SNARE synaptobrevin. Analysis of foot current amplitudes with synaptobrevin membrane anchor mutants are currently underway and are yielding results with clear parallels to those obtained with syntaxin (Hui, Jackson, and Chapman, unpublished data). Thus, a picture of the initial fusion pore of SNARE protein-mediated exocytosis is emerging in which rings of SNARE protein membrane anchors form the walls of the fusion pore. The two half channels through the vesicle and plasma membrane are then held together by the SNARE complex, a four-helix bundle formed by the cytoplasmic domains of the SNARE proteins (Fig. 3). The SNARE complex includes α-helices from syntaxin and synaptobrevin, and two additional α-helices from another t-SNARE, SNAP-25.

The SNARE complex plays a role in driving fusion pore transitions. Mutagenesis studies of the interior residues of the SNARE complex alter the rates of various kinetic steps initiated by Ca^{2+}. Thus, the initial frequency of fusion events and the maximal frequency of fusion events are altered by mutations in essentially every part of the SNARE complex. The fusion pore lifetime also changed with some of the SNARE protein mutations. Nearly all of these mutations prolonged the fusion pore lifetime, and most of them were closer to the membrane anchors. These results were interpreted as indicating that the SNARE complex starts to unravel as the fusion pore opens and dilates [23]. A sequence of transitions within the SNARE complex were proposed to drive the formation, opening, and dilation of the fusion pore, as illustrated in Fig. 3A–C.

At the present time a number of outstanding issues in exocytosis are controversial. For example, another recent study addressing the role of the SNARE complex came to the very different conclusion that the SNARE complex assembles vectorially from the N- to C-termini during Ca^{2+}-triggered exocytosis [24]. This study focused on the two helices contributed by SNAP-25 and used capacitance recording to follow the time course of exocytosis elicited by photolytic release of caged Ca^{2+} in chromaffin cells. Mutations in the N-terminal end had no effect, mutations in the middle inhibited an early priming step, and mutations near the C-terminal end inhibited a late triggering step. The interpretation of these data in terms of a vectorial assembly, or zipping, of the SNARE complex toward the membrane anchors of syntaxin and synaptobrevin is opposite to our interpretation of SNARE complex disassembly near the membrane anchors during fusion [23]. This issue remains to be resolved. Interestingly, the evidence for disassembly during a late step was based entirely on fusion pore lifetime data [23], where the time scale is on the order of 1 ms. The fastest process examined in the chromaffin

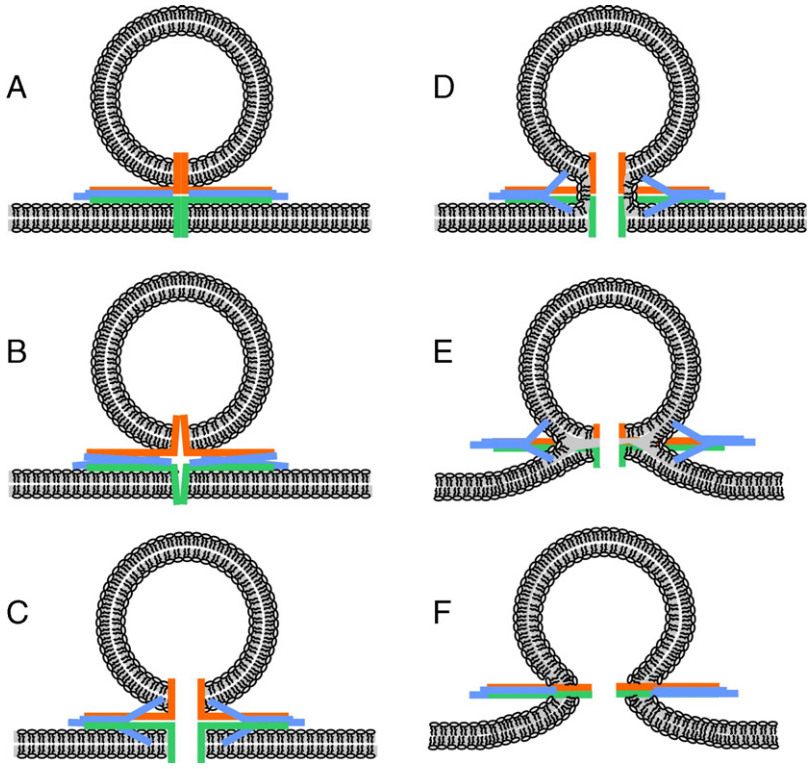

Fig. 3. Intermediates in exocytosis. (A) A SNARE complex tethers a vesicle to the plasma membrane. The membrane anchors of syntaxin (green) and synaptobrevin (orange) span the membranes and form a closed pore. SNAP-25 (blue) contributes two helices to the SNARE complex. (B) A rearrangement in the SNARE complex precedes fusion pore opening. (C) The opening of the fusion pore is accompanied by partial disassembly of the SNARE complex. (D) Lipid might flow around the open proteinaceous fusion pore. (E) Kinking the membrane anchors could facilitate lipid flux. (F) The end point of membrane fusion places the two SNARE membrane anchors in the same lipid bilayer.

cell study was a rapid capacitance rise, referred to as triggering, with a time scale on the order of 100 ms [24]. Thus, the disassembly step could occur after triggering. A sequential assembly of the SNARE complex as a vesicle progresses through a primed state to initiate triggering is still consistent with a subsequent disassembly implied by the fusion pore data in PC12 cells.

Another interesting point of controversy arises from experiments suggesting that the fusion pore provides a pathway for the flow of membrane from the vesicle to the cell surface [5] and that lipids can pass through the fusion pore [25]. Closely related to this is the work suggesting that fusion proceeds through a hemifusion intermediate [26,27]. This argues for a fusion pore composed of lipid rather than of protein as proposed by Han et al. [21,22]. However, these results are easily reconciled. A proteinaceous fusion pore could be surrounded by lipid in a way that allows the proximal bilayer leaflets to mix first (Fig. 3D). We know essentially nothing about the evolution of the fusion pore after it opens, and how the two membranes actually merge. Fig. 3E offers another speculative picture about how this might occur. The membrane anchors might bend sharply as the proximal leaflets mix. The membrane anchors of synaptobrevin and syntaxin have 3–4 glycines that could make the domains flexible and allow such a deformation. Ultimately, all of the membrane anchors end up in the same lipid bilayer after the fusion pore has finished its work (Fig. 3F), but we do not know the degree to which the SNARE motifs are associated at this point.

3. Fusion pores and synaptic transmission

As we learn more about fusion pores we can attempt to gain a clearer understanding of synaptic transmission. The initial size of the fusion pore and its rate of growth set limits on how rapidly transmitter can escape from a vesicle. This rate of neurotransmitter efflux then determines the time course of neurotransmitter concentration at the post-synaptic membrane. A considerable body of theoretical work has analyzed the process of transmitter release as diffusion through a fusion pore and into the synaptic cleft [28–32]. Most of this work treated release as instantaneous, or allowed the fusion pore to expand rapidly as it would if the vesicle were in the process of undergoing full fusion. If the fusion pore closes without dilating in a kiss-and-run action (a reverse transition from Fig. 3C back to B), then release will be determined by the rate of efflux through a relatively static structure. This allows the structure of a fusion pore to have a major physiological impact on the kinetics of release and on the time course of a synaptic response. In this final section some analytical solutions of the diffusion equation are examined to gain some insight into the profile of the transmitter concentration during kiss-and-run events.

When a fusion pore opens and connects the vesicle lumen with the synaptic cleft, the fusion pore acts as a circular source of transmitter, with transmitter diffusing radially outward and filling the synaptic cleft. Two views of this geometry are shown in Fig. 4. Circular geometry suggests that we can treat the problem as diffusion in two dimensions (Fig. 4A). Thus, the diffusion equation can be expressed in coordinates where the only relevant spatial dimension is r, the radial distance from the center of the fusion pore

$$D\left(\frac{\partial^2 C}{\partial r^2}+\frac{1}{r}\frac{\partial C}{\partial r}\right)=\frac{\partial C}{\partial t} \tag{2}$$

C is the transmitter concentration and D is the diffusion constant. At the outer edge of the circular synaptic cleft ($r=b$ in Fig. 4A), transmitter is rapidly diluted by the large surrounding volume, so we have an absorbing boundary where the concentration of transmitter is taken as zero. At the edge of the fusion pore, at $r=a$, we have a constant flux that determines the first derivative of C with respect to r.

In this two-dimensional representation we ignore the angle that specifies the direction taken by neurotransmitter as it diffuses away from the fusion pore. We also ignore the distance, z, which specifies the distance along a dimension perpendicular to the pre- and postsynaptic membrane surfaces. The neglect of the angle is perfectly reasonable as there is no reason to expect anisotropic diffusion in the synaptic cleft. However, z can only be neglected after transmitter has diffused away from the fusion pore opening, because the geometry is not two-dimensional when a molecule first emerges from the fusion pore. Fig. 4B illustrates this point, showing that diffusion in the z-direction matters in the immediate volume around the fusion pore. Diffusion only becomes two-dimensional when r exceeds a distance approximately equal to the width of the synaptic cleft (indicated as w in Fig. 4B). This point will enter into the analysis of two-dimensional diffusion shortly.

We will first examine a simple steady-state solution of Eq. (2) and then evaluate the time-dependent equation in order to ascertain the validity of the steady-state solution. This will yield

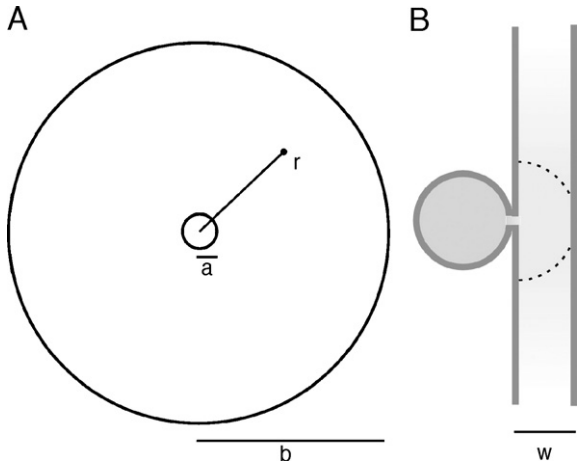

Fig. 4. The geometry of the fusion pore and synaptic cleft. (A) In the circular geometry, neurotransmitter diffuses radially outward from the fusion pore with radius a, and approaches the edge of the synapse, at a distance b from the center of the fusion pore. (B) A vesicle releases neurotransmitter into the synaptic cleft through a fusion pore. Close to the release site, neurotransmitter diffusion conforms to a roughly hemispherical geometry. At distances greater than the width of the synaptic cleft, w, the diffusion geometry is roughly circular, as depicted in (A).

an approximate expression for the synaptic cleft transmitter concentration during a kiss-and-run release event.

At steady-state, the time derivative of Eq. (1) equals zero, and the resulting differential equation in r is easily integrated. At $r=b$, the absorbing boundary condition sets $C=0$. At $r=a$, there is a constant flux, $F = -2\pi rwD\frac{\partial C}{\partial r}$. F is the flux through the fusion pore, in units of moles per second. This flux passes through the surface of a cylinder of length w and radius r. When $r=a$, this surface becomes the circular source in Fig. 4A, but this condition applies for any value of r between a and b. Applying these two boundary conditions to the steady-state solution of Eq. (2) gives

$$C(r) = \frac{F}{2\pi wD}\ln(b/r) \tag{3}$$

We cannot use this expression for $r<\sim w$ because the transmitter must diffuse across the synaptic cleft to reach the postsynaptic membrane. This relates to the point made above about the non-two-dimensional nature of the problem at the mouth of the fusion pore. This suggests that Eq. (2) will give the maximum steady-state concentration that can be reached at the postsynaptic membrane by setting $r=w=20$ nm [33] in Eq. (3).

Once the fusion pore has opened and transmitter has started to escape from the vesicle, the concentration will start to rise and approach the steady-state result just given. In order to know if the concentration will get this high we need to solve the full time-dependent expression (Eq. (1)), with the boundary conditions used to specify the steady-state solution (Eq. (2)), and with the initial condition $C(r,0)=0$. The solution to this problem is stated in Carslaw and Jaeger [34] at the end of Chapter 13.4.

$$C(r,t) = Fa\ln(b/r) + \pi F\sum_{n=1}^{\infty} e^{-D\alpha_n^2 t}\frac{J_o^2(b\alpha_n)(J_0(r\alpha_n)Y_1(a\alpha_n) - Y_0(r\alpha_n)J_0(r\alpha_n))}{\alpha_n(J_1^2(a\alpha_n) - J_0^2(b\alpha_n))} \tag{4}$$

where α_n are the roots to the equation

$$J_1(a\alpha)Y_0(b\alpha) - Y_1(a\alpha)J_0(b\alpha) = 0 \tag{5}$$

J_0, J_1, Y_0, and Y_1 are zero and first order Bessel functions of the first and second kind. Note that at $t=0$, the sum on the right gives a modified Fourier–Bessel representation of the steady-state solution, so that initially $C(r,0)=0$ as required. The exponentials decay with time so the solution goes over to the steady-state solution, as expected.

Eqs. (4) and (5) were evaluated with the computer program Mathcad 11 and the results are plotted in Fig. 5A for $a=1$ nm, $b=1$ μm, $D=7.6\times10^{-6}$ cm^2 s^{-1} [32], and $r=20$ nm (the smallest relevant value), 100 nm, and 500 nm. This plot shows that the concentration gets close to the steady-state level in ~200 μs for $r=20$ and 100 nm, and somewhat longer for $r=500$ nm. This agrees with a variety of numerical and Monte Carlo studies [30]. This time scale is fairly insensitive to most of the parameters selected here. Thus, the concentration profile rapidly reaches a steady state very soon after the fusion pore opens.

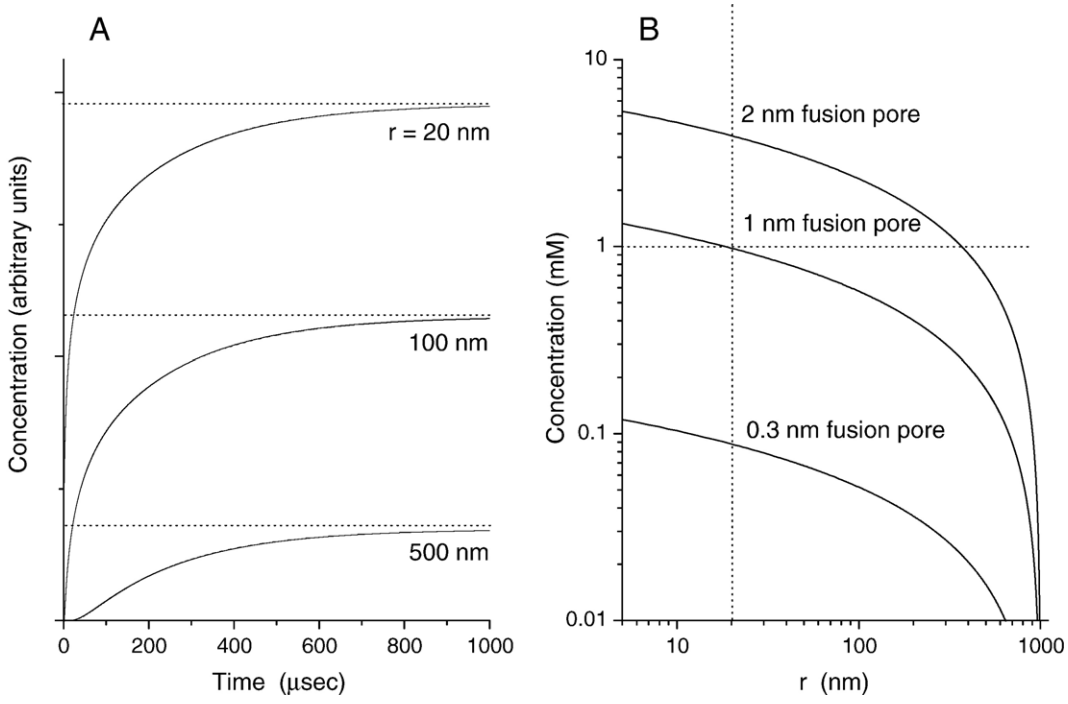

Fig. 5. The transmitter concentration in the synaptic cleft. (A) Evaluation of Eqs. (4) and (5) give the transmitter concentration as a function of time for different distances (r in Fig. 4A) from the center of the fusion pore. Dashed horizontal lines indicate final levels when the concentration has reached a steady-state. (B) Evaluation of Eq. (8) gives the steady-state concentration as a function of r for different fusion pore sizes. The vertical dotted line indicates the boundary of applicability of the circular geometry approximation. Values to the left are not relevant. The horizontal dotted line indicates the neurotransmitter level estimated in the synaptic cleft during synaptic transmission [30,36].

To compare this time to reach steady-state with the time that transmitter diffuses through an open fusion pore, note that diffusion through a fixed fusion pore predicts a nearly exponential decrease in the transmitter concentration within a vesicle, with a time constant of $\tau = V/(\rho D\gamma)$, where V is vesicle volume, ρ is saline resistivity, D is the transmitter diffusion constant, and γ is the fusion pore conductance [35]. For parameters relevant to a synaptic vesicle, this expulsion time was estimated as 5.7 ms [4]. For a typical dense core vesicle with a diameter of 200 nm and a fusion pore conductance of 250 pS, we get 23 ms. These expulsion times are much longer than the time it takes to reach a steady state in the synaptic cleft, so the transmitter concentration in the synaptic cleft will be well approximated by the steady-state solution in Eq. (3). The large difference between the time scale of equilibration within the cleft versus that for emptying the vesicle suggests that the concentration profile in the cleft will have the logarithmic form in Eq. (3). F decays exponentially, but at a slow rate compared to the time for transmitter to reach steady-state in the synaptic cleft, so we can envision this steady-state distribution decaying uniformly through the region as the transmitter in the vesicle dwindles. The maximum transmitter concentration will be the steady-state concentration with the initial flux seen immediately after the fusion pore opens.

Since a steady-state is reached quickly, plots of Eq. (3) give a useful profile of transmitter concentration. The only remaining quantity needed in order to use Eq. (3) to calculate the transmitter concentration in the synaptic cleft is the initial flux through a newly opened fusion pore. For an exponential dependence on time, the initial rate of transmitter loss is M_{ves}/τ, where M_{ves} is the moles of transmitter in a vesicle and τ was as specified above. Thus, we have

$$F = \frac{M_{ves}\rho D\gamma}{V} \tag{6}$$

Using Eq. (1) for γ, and replacing M_{ves}/V by the transmitter concentration in the vesicle, C_{ves}, we have

$$F = C_{ves} D\pi a^2/d \tag{7}$$

where d is the length of the fusion pore. We can now write Eq. (3) as

$$C(r) = \frac{C_{ves}a^2}{2dw}\ln(b/r) \tag{8}$$

Plotting Eq. (8) now can provide a view of the concentration of transmitter in the synaptic cleft (Fig. 5B). In all plots, $C_{ves} = 100$ mM, $d = 1$ nm (twice the thickness of a lipid bilayer), and $w = 20$ nm [33]. The plot with $a = 0.3$ nm gives the concentration profile expected for a 0.3 nm fusion pore, which is what we would expect based on the fusion pore conductance of vesicles of this type [4]. This shows that the concentration in the synaptic cleft reaches a bit less than 100 μM, which is well below the experimental value of 1 mM [33,36]. In synapses where kiss-and-run exocytosis employs this type of fusion pore, the concentration of transmitter in the synaptic cleft will be so low that the low-affinity AMPA receptors will be weakly activated, if at all.

For a larger fusion pore of about the size seen with a dense-core vesicle, 1 nm, the concentration just barely reaches the critical 1 mM level, and for a 2 nm fusion pore, which is a size only seen after quite a bit of expansion [37,38], the concentration surpasses that estimated from experiment. With a fusion pore this large the transmitter decay time constant works out to ~0.5 ms, so the steady-state approximation starts to break down. The cleft concentration would be higher than for a 1 nm fusion pore, but it would not reach the level indicated in Fig. 5B.

This analysis indicates the rapid synaptic responses that have been attributed to kiss-and-run [39] require a fusion pore much larger than that seen in experiments with vesicles closely related to synaptic vesicles. A Monte Carlo analysis of transmitter diffusion at rapid excitatory synapses, which also considered receptor kinetics, indicated that a fusion pore radius of 3.5 nm is necessary to produce the observed time course of a synaptic current [32]. Since the small fusion pores seen during kiss-and-run and during the earliest stages of full fusion cannot support rapid synaptic responses, a mechanism is required to allow fusion pores to expand very rapidly. This theoretical analysis helps bring this process into focus, and should stimulate efforts to characterize fusion pores in greater detail with higher time resolution.

The properties of fusion pores must dictate what can happen at synapses. In the *Drosophila* neuromuscular junction presynaptic manipulations altered the time course of quantal events in a manner consistent with effects on fusion pores [40]. There are very few such studies and more work along these lines will be of great value in relating the results obtained in model systems such as PC12 cells to the mechanisms that operate in an intact synapse.

Acknowledgements

I thank Vitaly Klyachko, Camin Dean, and Ed Chapman for helpful discussions and comments on this manuscript. This work is supported by NIH grants NS30016 and NS44057.

References

[1] J.E. Heuser, T.S. Reese, Structural changes after transmitter release at the frog neuromuscular junction, J. Cell Biol. 88 (1981) 564–580.
[2] E. Neher, A. Marty, Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 6712–6716.
[3] J.M. Fernandez, E. Neher, B.D. Gomperts, Capacitance measurements reveal stepwise fusion events in degranulating mast cells, Nature 312 (1984) 453–455.
[4] V.A. Klyachko, M.B. Jackson, Capacitance steps and fusion pores of small and large-dense-core vesicles in nerve terminals, Nature 418 (2002) 89–92.
[5] R. Monck, G. Alvarez de Toledo, J.M. Fernandez, Tension in secretory granule membranes causes extensive membrane transfer through the exocytotic fusion pore, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 7804–7808.
[6] M. Lindau, W. Almers, Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion, Curr. Opin. Cell Biol. 7 (1995) 509–517.

[7] B. Hille, Ion Channels of Excitable Membranes, Sinauer Associates, Sunderland, 2001.
[8] M.B. Jackson, Molecular and Cellular Biophysics, Cambridge University Press, Cambridge, 2006.
[9] R.H. Chow, L. von Rüden, E. Neher, Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells, Nature 356 (1992) 60–63.
[10] C.T. Wang, R. Grishanin, C.A. Earles, P.Y. Chang, T.F. Martin, E.R. Chapman, M.B. Jackson, Synaptotagmin modulation of fusion pore kinetics in regulated exocytosis of dense-core vesicles, Science 294 (2001) 1111–1115.
[11] W.D. Matthew, L. Tsavaler, L.F. Reichardt, Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue, J. Cell Biol. 91 (1981) 257–269.
[12] M.S. Perin, V.A. Fried, G.A. Mignery, R. Jahn, T.C. Sudhof, Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C, Nature 345 (1990) 260–263.
[13] E.R. Chapman, Synaptotagmin: a Ca^{2+} sensor that triggers exocytosis? Nat. Rev., Mol. Cell Biol. 3 (2002) 498–508.
[14] R. Jahn, T. Lang, T.C. Sudhof, Membrane fusion, Cell 112 (2003) 519–533.
[15] T.W. Koh, H.J. Bellen, Synaptotagmin I, a Ca^{2+} sensor for neurotransmitter release, Trends Neurosci. 26 (2003) 413–422.
[16] W.C. Tucker, J.M. Edwardson, J. Bai, H.J. Kim, T.F. Martin, E.R. Chapman, Identification of synaptotagmin effectors via acute inhibition of secretion from cracked PC12 cells, J. Cell Biol. 162 (2003) 199–209.
[17] D. Colquhoun, A.G. Hawkes, On the stochastic properties of bursts of single ion channel openings and of clusters of bursts, Philos. Trans. R. Soc. Lond., B Biol. Sci. 300 (1982) 1–59.
[18] M.B. Jackson, Ion channels. Single-channel analysis, Methods Enzymol. 207 (1992) 729–746.
[19] J. Bai, C.T. Wang, D.A. Richards, M.B. Jackson, E.R. Chapman, Fusion pore dynamics are regulated by synaptotagmin∗t-SNARE interactions, Neuron 41 (2004) 929–942.
[20] C.T. Wang, J. Bai, P.Y. Chang, E.R. Chapman, M.B. Jackson, Synaptotagmin-Ca^{2+} triggers two sequential steps in regulated exocytosis in rat PC12 cells: fusion pore opening and fusion pore dilation, J. Physiol. 570 (2006) 295–307.
[21] X. Han, C.T. Wang, J. Bai, E.R. Chapman, M.B. Jackson, Transmembrane segments of syntaxin line the fusion pore of Ca^{2+}-triggered exocytosis, Science 304 (2004) 289–292.
[22] X. Han, M.B. Jackson, Electrostatic interactions between the syntaxin membrane anchor and neurotransmitter passing through the fusion pore, Biophys. J. 88 (2005) L20–L22.
[23] X. Han, M.B. Jackson, Structural transitions in the synaptic SNARE complex during Ca^{2+}-triggered exocytosis, J. Cell Biol. 172 (2006) 281–293.
[24] J.B. Sorensen, K. Wiederhold, E.M. Muller, I. Milosevic, G. Nagy, B.L. de Groot, H. Grubmuller, D. Fasshauer, Sequential N- to C-terminal SNARE complex assembly drives priming and fusion of secretory vesicles, EMBO J. 25 (2006) 955–966.
[25] J.W. Taraska, D. Perrais, M. Ohara-Imaizumi, S. Nagamatsu, W. Almers, Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 2070–2075.
[26] Y. Xu, F. Zhang, Z. Su, J.A. McNew, Y.K. Shin, Hemifusion in SNARE-mediated membrane fusion, Nat. Struct. Mol. Biol. 12 (2005) 417–422.
[27] C. Reese, F. Heise, A. Mayer, Trans-SNARE pairing can precede a hemifusion intermediate in intracellular membrane fusion, Nature 436 (2005) 410–414.
[28] J.C. Eccles, J.C. Jaeger, The relationship between the mode of operation and the dimensions of the junctional regions at synapses and motor end-organs, Proc. R. Soc. Lond., B 148 (1958) 38–56.
[29] R. Khanin, H. Parnas, L. Segel, Diffusion cannot govern the discharge of neurotransmitter in fast synapses, Biophys. J. 67 (1994) 966–972.
[30] J.D. Clements, Transmitter timecourse in the synaptic cleft: its role in central synaptic function, Trends Neurosci. 19 (1996) 163–171.
[31] J.R. Stiles, D. Van Helden, T.M. Bartol, E.E. Salpeter, M.M. Salpeter, Miniature endplate current rise times <100 ms from improved dual recordings can be modeled with passive acetylcholine diffusion from a synaptic vesicle, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5747–5752.
[32] L.M. Wahl, C. Pouzat, K.J. Stratford, Monte Carlo simulation of fast excitatory transmission at a hippocampal synapse, J. Neurophysiol. 75 (1996) 597–608.
[33] F.A. Edwards, Anatomy and electrophysiology of fast central synapses lead to a structural model for long-term potentiation, Physiol. Rev. 75 (1995) 759–787.
[34] H.S. Carslaw, J.C. Jaeger, Conduction of Heat in Solids, 2nd ed. Clarendon Press, Oxford, 1959.
[35] W. Almers, L.J. Breckenridge, A. Iwata, A.K. Lee, A.E. Spruce, F.W. Tse, Millisecond studies of single membrane fusion events, Ann. N. Y. Acad. Sci. 635 (1991) 318–327.
[36] J.D. Clements, R.A. Lester, G. Tong, C.E. Jahr, G.L. Westbrook, The time course of glutamate in the synaptic cleft, Science 258 (1992) 1498–1501.
[37] A.E. Spruce, L.J. Breckenridge, A.K. Lee, W. Almers, Properties of the fusion pore that forms during exocytosis of a mast cell secretory vesicle, Neuron 4 (1990) 643–654.
[38] N. Takahashi, T. Kishimoto, T. Nemoto, T. Kadowaki, H. Kasai, Fusion pore dynamics and insulin granule exocytosis in the pancreatic islet, Science 297 (2002) 1349–1352.
[39] C.F. Stevens, J.H. Williams, “Kiss and run” exocytosis at hippocampal synapses, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 12828–12833.
[40] C. Pawlu, A. DeAntonio, M. Heckmann, Postfusional control of quantal current shape, Neuron 42 (2004) 607–618.
[41] X. Han, PhD thesis Department of Physiology p. 163, University of Wisconsin, Madison, 2004.