Adrenaline Release by Chromaffin Cells: Constrained Swelling of the Vesicle Matrix Leads to Full Fusion**

Christian Amatore,* Yann Bouret, Eric R. Travis, and R. Mark Wightman

Release of neurotransmitters by secretory vesicles is a fundamental neurobiological process but some of its physicochemical basis remains a mystery. In particular, the reason is still unknown why some vesicles proceed to a stage involving full inclusion of their former membrane into that of the cell (full fusion), whereas others remain poised at the initial stage of the process, where release occurs through a nanometer-sized pore (fusion pore).

In this paper, we investigate quantitatively this problem by examining the kinetic information contained in the experimental dynamics of the vesicle membrane during the full-fusion stage. We take advantage of our recent work, in which we established quantitatively that the dynamics of full fusion can be determined with unprecedented precision through deconvolution of chronoamperometric currents during individual exocytotic secretion events measured relative to release of adrenaline by chromaffin cells.[1] This treatment demonstrated that full fusion secretion events are regulated by the interplay between the swelling of the core of the vesicle and the dynamics of the membrane unfolding although the efflux of catecholamine cations (adrenaline, in this work) remains limited kinetically by its slow diffusion inside the exposed matrix. The present work affords a quantitative description of the physicochemical forces regulating the transition between fusion-pore and full-fusion states. During the pore-release phase, the internal vesicle matrix is confined by its membrane, so it cannot expand significantly. This restrained swelling of the confined matrix drastically weakens the stability of the fusion pore by increasing the membrane tension. When the surface tension forces overcome its edge resistance, the fusion pore becomes unstable and expands rapidly, thereby initiating full fusion. This concept implies that very small vesicles should never reach the transition point because they cannot develop a sufficient membrane surface tension due to an insufficient internal swelling pressure, whereas large vesicles should always proceed to the full-fusion stage and their matrix is thereby fully exposed to the extracellular fluid at the end of the process.

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In chromaffin cell vesicles, catecholamine cations are tightly associated with a negatively charged matrix composed of chromogranins and ATP. Because of electroneutrality, catecholamine cations can only escape upon exchange with hydrated monovalent cations from the extracellular solution.[2] As reported for synthetic hydrogels,[3, 4] this exchange disrupts hydrogen bonds and creates weak dipole–dipole repulsion in the matrix structure and thereby imparts upon it a tendency to swell.[5–7] For example, the radius of mast cell granules is reported to expand by a factor of about 1.35 upon full release.[6, 7] Swelling increases ionic diffusivity in the matrix, which allows further penetration of solvated ions and eventually leads to a cascade of released catecholamine cations. However, the matrix swelling is initially impeded by the tight cellular membrane and the mechanism of catecholamine cation release is necessarily more complex.

Exocytosis of catecholamines by chromaffin cells can be measured with a carbon-fiber ultramicroelectrode placed adjacent to the cell.[8–11] This ensures the electrooxidation (two electrons per molecule[12]) of secreted molecules that diffuse from the cell to the electrode. The released catecholamine flux $\frac{dN(t)}{dt}$, where $N(t)$ is the number of catecholamine molecules released at time t, is converted into a current $I(t)$ according to Faraday's law: $I(t) = 2F\frac{dN(t)}{dt}$, where F is the Faraday constant. The variation of the current with time (Figure 1) is thus a direct kinetic measurement of the catecholamine efflux. Simultaneous amperometric and patch–clamp investigations demonstrated that release starts through the formation of a fusion pore that connects the inside of the vesicle to the extracellular fluid through the two contacting membranes.[13–15] Any event prior to the formation of the initial fusion-pore aperture is not considered here, since our method gives only access to the rate of efflux of catecholamine cations. For most chromaffin vesicles, this pore-release phase proceeds to the full fusion of vesicle and cell membrane.[13–15]

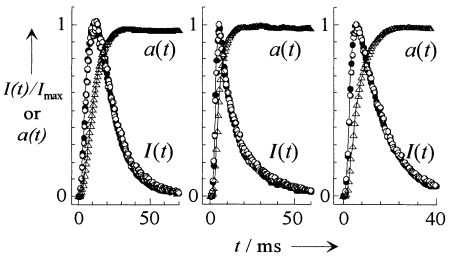

Figure 1. Normalized chronoamperometric spikes $I(t)/I_{max}$, measured as described previously,[26, 27] and the variations of $a(t)$[1] for three representative individual exocytotic events observed at chromaffin cells stimulated to release adrenaline upon the addition of Ba^{2+}.[26, 27] $I(t)$: Current at time t; I_{max}: Maximum current of each event; $a(t)$: Fraction of the vesicle surface area exposed to the extracellular fluid at some time t after the beginning of full fusion (see Figure 2). Experimental (open circles) and theoretical (solid circles) currents based on the extracted $a(t)$ functions (open triangles).[1]

A steady flux during the pore lifetime is observed by chronoamperometry as a small constant current, termed I_{foot} (about 5 pA, merged with the initial current rise in Figure 1).

Transition to the full-fusion stage results in a rapid current increase that decays within a few milliseconds.[8–11, 13, 14] As established previously,[11] such a sharp spike is inconsistent with the matrix swelling rate alone controlling the kinetics,[3] in contradiction to previous claims.[7] Rather, the current spike is consistent with convoluted kinetics involving the dynamics of the vesicle membrane and the rate of diffusion of catecholamine out of the matrix.[1] Application of this model to a large number of chronoamperometric spikes allowed its experimental validation,[1] as well as measurement of catecholamine concentration and diffusion inside the swollen matrix.[16] Extraction of the diffusive kinetic components thereby afforded the vesicle-membrane dynamics with unprecedented precision.[1, 16]

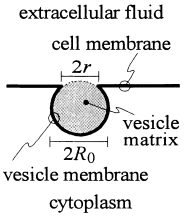

Figure 2. Schematic representation of the vesicle and cell membranes during full fusion, with a definition of the vesicle radius R_0 and the expanding pore radius r.

The surface area function $a(t)$ extracted from the current trace[1] (Figure 1) represents the fractional area of the spherical vesicle matrix of radius R_0 (Figure 2) exposed to the extracellular fluid at time t after the beginning of the full-fusion event. In agreement with patch–clamp capacitance measurements,[13, 14] $a(t)$ has an approximately sigmoidal shape (see triangles in Figure 1). However, the present precision demonstrates that $a(t)$ exhibits a profound asymmetry between short- and long-term behaviors. During the initial period, $a(t)$ increases explosively, yet later it increases at a much smoother pace. We have suggested that this arises because different physicochemical factors control the vesicle membrane dynamics.[1] We demonstrate herein that this intuitive claim is quantitatively supported.

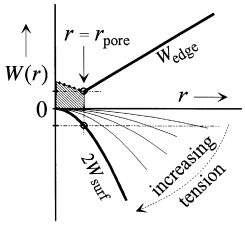

Figure 3. Schematic representation of the edge potential energy $W_{edge} = 2\pi\rho_0 r$ and the surface-tension potential energy $W_{surf} = -\pi\sigma r^2$ as a function of the pore radius r. The component W_{surf} is represented for different surface-tension energy coefficients (σ) to illustrate the effect of an increase in this parameter due to the increasing internal pressure in the vesicle. The shadowed box symbolizes the edge energy of the initial fusion-pore structure that opposes its closure (see text).

The full-fusion event can be described as a pore-enlargement process.[16] In a membrane, a circular pore of radius r has a potential energy $W(r)$, which is the sum of two components,[17] $W(r) = W_{surf} + W_{edge}$ (see Figure 3). The term $W_{surf} = -\pi\sigma r^2$ represents the surface-tension work which tends to enlarge the pore radius (the surface-tension energy coefficient σ, for a differential pressure ΔP between the membrane surfaces, is given by $\sigma = \frac{1}{2}\Delta P R_0$) and the term $W_{edge} = 2\pi\rho_0 r$ represents the potential energy of the pore edge that opposes its enlargement (ρ_0 is the edge energy coefficient). The value of W_{edge} during the pore-release phase, represented by the shaded box in Figure 3, is determined by biological processes that are still not fully characterized. In any case, during this initial phase, the overall energy has a minimum at $r = r_{pore} =$ ~ 1 nm, since initial fusion pores are known to have this radius.[11, 13, 14, 18] Since these pores are reported to flicker ($0 \le r \le r_{pore}$) in phase with thermal fluctuations,[13–15, 18] the value of $W_{edge}(r_{pore})$ must be comparable to $k_B T$ (k_B is Boltzmann's constant).

During the pore-release stage, $W_{edge}(r_{pore}) > -W_{surf}(r_{pore})$ (Figure 3). However, during this stage, a steady-state flux of catecholamine cations exchanges with hydrated cations from the extracellular fluid. This results in a continuous modification of the matrix near the fusion pore and an increasing drive for these altered matrix zones to swell.[5–7] While the vesicle is constricted by its membrane, it cannot swell significantly and thereby ΔP and the surface-tension coefficient σ increase.[19] The tightening of the vesicle membrane proceeds up to the moment when the initial fusion pore becomes metastable,[17] a situation requiring $dW(r)/dr = 0$, that is, $\Delta P_{rupt} = 2\sigma_{rupt}/R_0 = 2\rho_0/r_{pore}R_0$ (ΔP_{rupt} and σ_{rupt} are the pressure and surface-tension coefficient at the moment of pore rupture). Any further alteration of the matrix provokes the pore rupture. Provided that the membrane tension does not relax significantly, during the rupture phase the pore must enlarge irreversibly since $W_{surf}(r)$ varies with r^2 but $W_{edge}(r)$ varies only with r (see Figure 3).[17] It is beyond the scope of this work to propose a precise relationship between σ and r during the pore expansion stage. However, it seems realistic to consider that σ should not vary significantly while the membrane tightens the matrix, that is, the boundary between the cell and vesicle membranes has not yet reached the equatorial plane of the vesicle (Figure 4). Conversely, when more than half of the matrix is already exposed, it may freely expand towards the extracellular fluid and the membrane tension should relax significantly, and thus $\sigma \le \sigma_{rupt}$. This intuitive view[1] suggests that the pore edge energy can be neglected ($W(r) \approx -\pi\sigma r^2$, where $\sigma \approx \sigma_{rupt}$) during the first half of the full-fusion process but it dominates ($W(r) \approx +2\pi\rho_0 r$)[20] during the second half.[16] This imposes an accelerated expansion up to the matrix equatorial plane. Afterwards, the pore closes irreversibly, being tantamount to resorbing the remaining vesicle cupola on which the vesicle rested (Figure 4). The two phases occur at different speeds because of the different physicochemical factors involved. This view[1] explains qualitatively the asymmetric shape of the experimentally measured $a(t)$ function.

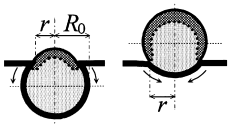

Figure 4. Schematic representation of the different topologies before and after the equatorial plane of the vesicle has been passed during the full-fusion process. The symbols are identical to those used in Figure 2, with the unaltered zones of the matrix shown lightly shaded and the swollen zones darker.

To proceed quantitatively, we need to investigate the dynamics of the full-fusion process. We suppose that $W(r)$ is converted into viscous dissipation, Equation (1), where η_s is

$$4\pi\eta_s r(dr/dt) = -W(r) \quad (1)$$

the surface viscosity.[21] We will consider the two halves of the full-fusion process separately since they obey different driving

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forces. Equation (1) predicts that during the first half, the radius should expand exponentially at $r = r_{pore}\exp(\sigma t/4\eta_s)$, which is clearly inconsistent with our experimental data: Figure 5a shows that the pore radius[22] expands at an initial rate comparable to the average rate over the entire time interval, rather than being comparatively negligible if an exponential growth occurred, as is usual in pore-nucleation processes.[21]

This establishes that surface-tension potential energy cannot be solely responsible for the rapid expansion of the initial pore. Our results are consistent with the additional dissipation of a constant energy E_0 during this process.[23] Let γ be the value of E_0 relative to $4\pi\sigma R_0^2$, the entire surface-tension potential energy immediately after the rupture; Equation (1) thereby affords Equation (2). Integration of Equation (2)

$$2(r/R_0)\frac{d(r/R_0)}{dt} = (\sigma/2\eta_s)[(r/R_0)^2 + 4\gamma] \quad (2)$$

gives $(r/R_0) = 2\gamma^{1/2}[\exp((\sigma t)/(2\eta_s)) - 1]^{1/2}$. Figure 5b shows that this prediction agrees extremely well with the experimental measurements (Figure 5a). This is further confirmed by the remarkably good correlation shown in Figure 5c, which includes all of the 232 events which could be treated at this stage of the study. This treatment showed that $\gamma \approx 0.005$ and thus the mean value of E_0 is a minute amount of the entire surface-tension energy at the beginning of full fusion. This is, nevertheless, a few hundred k_BT,[23] which explains its considerable role on the initial pore expansion rate.

When the matrix is already more than half exposed, the main driving force stems from the pore edge, $W(r) = +2\pi\rho_0 r$. Still supposing the viscous dissipation mode, Equation (1) transforms to Equation (3). This predicts that the pore radius

$$\frac{d(r/R_0)}{dt} = -\frac{\rho_0}{2R_0\eta_s} \quad (3)$$

decreases linearly with time $(r \propto -(\rho_0/2\eta_s)t)$, as recently observed for transient pore closure,[21] albeit in a very different context. This prediction agrees strikingly with the experimental variations observed for all the 596 experimental spikes treated in this study (Figure 6). The statistical treatment of these data revealed that the mean value of $2R_0\eta_s/\rho_0$ is about 7 ms. This corresponds to an average closure rate of 20 $\mu m\,s^{-1}$, which seems realistic for a weakly stressed membrane.[21]

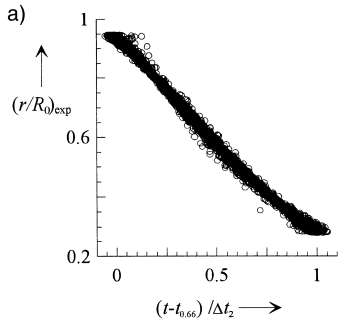

Figure 6. Correlation between theoretical predictions [Eq. (3)] and experimental variations of $r(t)/R_0$ during the second half of the full-fusion process (see the right panel of Figure 4; all of the 596 events are treated here; correlation coefficient 0.998); Δt_2 is the time required for $a(t)$ to increase from 0.66 to 0.98; $t_{0.66}$ is the time at which $a(t) = 0.66$.

The above two physicochemical and kinetic analyses and their remarkable quantitative agreement with the experimental measurements demonstrate that the dynamics of the full-fusion process is controlled by two different mechanisms, depending on the system topology. The initial events, when the matrix is still constricted by the vesicle membrane, are dictated by the surface-tension energy due to the pressure created by the restricted swelling of the vesicle. Thus, in agreement with previous conjecture by Neher on secretion without full fusion,[15] this model indicates that small vesicles cannot achieve full fusion because they cannot develop a sufficient pressure for their membrane surface tension to overcome their pore edge resistance. Conversely, larger vesicles should always proceed irreversibly to full fusion unless the pore closes by another mechanism before its rupture point is reached (namely, as in "kiss and run" events[14, 15]).

For the chromaffin cells investigated here, the mean initial fusion-pore duration is $t_{rupt} = (2.2 \pm 0.2)$ ms.[1, 16] Since I_{foot} is about 5 pA, this establishes, by Faraday's law, that to reach the rupture point, about 20000 catecholamine molecules among the 3–5 million that the matrix contains must be released.[11, 16] Since the mean value of R_0 is about 150 nm,[24] the depleted volume due to catecholamine efflux at t_{rupt} corresponds approximately to that of a sphere of about 25 nm radius. Assuming that the energetics of the present

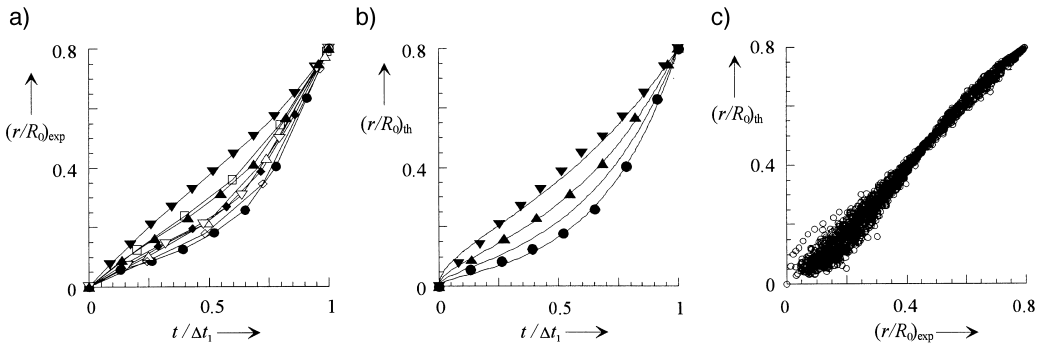

Figure 5. Variations of $r(t)/R_0$,[22] the full-fusion pore radius relative to that of the vesicle during the first half of the full-fusion process (see the left panel of Figure 4). a) Experimental variations for eight representative events; Δt_1 is the time for which $r(\Delta t_1) = 0.8\,R_0$. b) Theoretical predictions by Equation (2) for (from top to bottom) $\lg\gamma = -2$, -2.5, -3, and -3.5. Three representative events shown in (a) are overlaid (with the same symbols) to illustrate the remarkable agreement. c) Correlation between theoretical predictions for $r(t)/R_0$ [Eq. (2)] and experimental measurements for all of the 232 events which could be treated here (correlation coefficient 0.993).

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vesicles can be extrapolated to other catecholaminergic vesicles,[25] vesicles containing less than about 20000 molecules and with radii less than about 25 nm (such as small synaptic vesicles) should never reach full fusion and thereby be released only through their fusion pores. Conversely, larger ones (such as large, dense core vesicles) should always fully fuse unless another mechanism closes the pore before about 20000 molecules have been released. It is interesting that these values correspond precisely to the molecule content ($\sim$10000) and size (20–30 nm) of many catecholaminergic neuronal vesicles. In this perspective, these vesicles would represent the optimum balance between a nonfusional release and a maximum load of neurotransmitter.

Experimental Section

All experiments and procedures were identical to those previously described, either for the stimulation of chromaffin cells and monitoring of chronoamperometric traces,[26, 27] or for the deconvolution of the chronoamperometric data.[1, 16] In this study we treated 596 representative events. All events were used in the kinetic treatment of the second half of the full-fusion stage. However, to perform a significant kinetic treatment of the initial half of full fusion, several of these events could not be used, either because insufficient data points (<5) were available in the time range of interest or because residual foot features altered the spike rise. This reduced the series to 232 meaningful events, but the data presented here are consistent with all the discarded events.

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[20] For simplicity, we still use the same notation ρ_0 for the edge-energy coefficient of the pore, although the pore considered during the full-fusion stage is necessarily bilipidic and may differ from the initial fusion pore. However, since the value of $W_{edge}(r_{pore})$ has been estimated above to be in the range of k_BT, that is, close to that of a bilipidic pore of identical size (1 nm), in practical terms the two coefficients are similar even if the two pore structures may differ.
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[22] The pore radius $r(t)$ is readily obtained from the experimental relative surface area $a(t)$ by $r(t)/R_0 = \{1-[1-2a(t)]^2\}^{1/2}$.
[23] The use of a simple pore model in our treatment neglects the fact that the connection of the cell and vesicle membranes creates a "bottle neck" invagination. Therefore, a negative additional energy (termed the Gaussian curvature energy; see, for example: U. Seifert, R. Lipowsky in *Structure and Dynamics of Membranes* (Eds.: R. Lipowsky, E. Sackmann), Elsevier, Amsterdam, **1995**, pp. 409–411, must be added to $W(r)$. The modulus of Gaussian curvature energies is predicted to be about 300 k_BT, which compares excellently with the mean value of $E_0 \approx 120\ k_BT$ obtained here. Note that considering that the edge energy of the initial fusion pore is 2.5 k_BT, as is implicitly assumed here, would afford exactly $E_0 = 300\ k_BT$.
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Giant Artificial Ion Channels Formed by Self-Assembled, Cationic Rigid-Rod β-Barrels**

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Dedicated to Professor Koji Nakanishi on the occasion of his 75th birthday

The interior of toroidal biomacromolecules is a privileged site for molecular recognition, translocation, and transformation.[1–4] Giant ion channels (or nanopores) are a particularly

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