Function suggests nano-structure: towards a unified theory for secretion rate, a statistical mechanics approach

Ilan Hammel1 and Isaac Meilijson2

1Sackler Faculty of Medicine, Department of Pathology, and 2Raymond and Beverly Sackler Faculty of Exact Sciences, School of Mathematical Sciences, Department of Statistics and Operations Research, Tel Aviv University, Tel Aviv 6997801, Israel

The inventory of secretory granules along the plasma membrane can be viewed as maintained in two restricted compartments. The release-ready pool represents docked granules available for an initial stage of fast, immediate secretion, followed by a second stage of granule set-aside secretion pool, with significantly slower rate. Transmission electron microscopy ultrastructural investigations correlated with electrophysiological techniques and mathematical modelling have allowed the categorization of these secretory vesicle compartments, in which vesicles can be in various states of secretory competence. Using the above-mentioned approaches, the kinetics of single vesicle exocytosis can be worked out. The ultra-fast kinetics, explored in this study, represents the immediately available release-ready pool, in which granules bound to the plasma membrane are exocytosed upon Ca2+ influx at the SNARE rosette at the base of porosomes. Formalizing Dodge and Rahamimoff findings on the effect of calcium concentration and incorporating the effect of SNARE transient rosette size, we postulate that secretion rate (rate), the number (\(X\)) of intracellular calcium ions available for fusion, calcium capacity (0 \(\leq M \leq 5\)) and the fusion nano-machine size (as measured by the SNARE rosette size \(K\)) satisfy the parsimonious M–K relation: rate \(\approx C \times [\text{Ca}^{2+}]^{m} \times m^{m} \times e^{-K/2}\).

1. Introduction

Exocytic events of release-ready vesicle pool from secretory cells [1–3] follow a time course consistent with calcium concentration [4] and with Poisson behaviour [5,6], as evidenced by a number of statistical measures, such as the estimate of its rate, the number of events recorded per unit time [6]. For vesicular secretion, this direct measure exhibits a variety of statistical properties that can be monitored. In particular, the probability of granule fusion with the plasma membrane, which establishes the rate of secretion, is highly dependent on random SNARE protein aggregation in a rosette at the porosome base [5], arrival time and biochemical activity [1–6]. Based on statistical mechanics modelling composed with meta-analysis of experimental data, we propose a generalization of the common formula [4] for secretion rate, rate \(= C \times [\text{Ca}^{2+}]^{M}\), expressed in terms of two key parameters that dictate secretion rate, calcium concentration [\(\text{Ca}^{2+}\)] and the (maximal) calcium cooperativity (integer) coefficient \(M\) (0 \(\leq M \leq 5\)), into the M–K relation: rate \(\approx C_{1} \times E([C_{2} \times [\text{Ca}^{2+}]^{M}])^{m} \times e^{-K/2}\), where \(K\) is the (granule size-dependent) number of SNAREs to form the secretion rosette, and \(X\) is the (possibly random, calcium concentration-dependent) actual cooperativity coefficient. Syaptic vesicle release occurs spontaneously in the absence of Ca2+ binding at the calyx of Held [7], in spite of low secretion rate and thus \(K\) can be 0. In previous work, we have determined that \(K\) must be at least 1 [5].

Cellular communication depends on membrane fusion mechanisms. Fusion of a secretory granule with the porosome at the cell plasma membrane [6–10] is considered the critical step in regulated exocytosis. Granule fusion events, commonly quantified as a Poisson process [6,7], are controlled by the interplay...
between cellular Ca\(^{2+}\) signals and catalysed by specific proteins (mainly SNAREs) [1–11]. The delivery of newly formed secretory content to the cytoplasm granule inventory occurs through a direct fusion of recently formed granules and mature granules. The authors have documented [12–14] evidence that newly formed granules are monomers of quantal size \(G_1\) and larger granules are polymer (\(n\)-mer) progressive aggregates of the granule monomer \((G_n = nG_1)\). SNARE proteins play an essential role in all intracellular fusion reactions associated with the life cycle of secretory vesicles, whether vesicle–vesicle (homotypic fusion, as just described) or vesicle–plasma membrane fusion (heterotypic fusion, leading to secretion).

2. The model

2.1. Dependence on the SNARE rosette petals

The authors have developed a growth and elimination (G&E) model that describes a possible mechanism for basal granule growth and secretion [5,16]. According to this model, homogeneous unit granules are packaged in the Golgi apparatus (or otherwise), with some mean content (\(i.e., \text{volume}\)) \(\nu\) and standard deviation \(\sigma\). Granules grow by fusing with freshly packaged unit granules, or exit the cell. The rate at which a granule of type \(G_n\) becomes of type \(G_{n+1}\) is \(\lambda_n = \nu \mu_n\) and the rate at which the granule exits the cell is \(\mu_n\). As usual with Markov modelling, these two competing scenarios manifest themselves at independent exponentially distributed times, and the earliest to happen, wins. The authors have argued [5,16] that \(\gamma = - (2/3)(K_\beta - 1)\) and \(\beta = - (2/3)(K_\beta - 1)\), where \(K_\beta\) is the number of SNARE protein complexes that constitute the rosette that docks a granule with the cell membrane for exocytosis, and \(K_\beta\) is a physical or conceptual counterpart for homotypic fusion of a mature granule and a unit granule. This model explains the quantal nature of granule content, both in the cytoplasm and upon one-granule-at-a-time secretion. Quantal means [14–16] that granules of type \(G_n\) have mean content \(\nu\) and standard deviation of content \(\sigma\). Thus, quantal granule content distribution is multimodal, with equally spaced peaks. The measured standard deviation of the \(n\)th peak should behave such as \(\sqrt{n \sigma^2 + \tau^2}\), where \(\tau\) is the error of measurement standard deviation [14–16]. The packaging coefficient of variation \(\sigma/\nu\) is of the order of 10\(^{-9}\), and the error of measurement coefficient of variation is of the order of 10–30\% [14–16]. A distinctive feature of the G&E model is that it predicts the emergence of two distinct granule volume distributions, the stationary distribution that describes steady-state statistics in the cytoplasm and the exit distribution that describes granule content distribution at secretion [5,16]. These two distributions carve up quantal nature, share the parameters \(\nu, \sigma\) and \(\tau\) but differ in the mixture weights at the various peaks. This statistical mechanics approach views SNARE components as interacting particles, and provides a simple mathematical explanation for a steady-state ‘fusion nano-machine’ of SNARE self-aggregation, that predicts secretion rates as described above, proportional to a term of the form \(C_5\), as in the \(M-K\) rate relation above. Based on simple Euclidean geometry, the authors have argued [9] that the SNARE rosette size \(K\) should be proportional to the square root of granule diameter, and estimated it empirically from the mean granule diameter \((D, \text{nm})\) as \(K_y = (0.9 \pm 0.2) \sqrt{D}\). Statistical properties of evoked exocytic events were checked over a wide range of granule sizes \((D = 25–1270 \text{ nm}, \nu = 8 \times 10^3 - 1.1 \times 10^8 \text{ nm}^3\)\), using secretion rates and granule morphometric characteristics recorded from published data (see electronic supplementary material, table S1). Scattergram analysis of the natural logarithm of secretion burst rate versus estimated rosette size confirms a linear relation (figure 1), leading very close to rate \(\approx 4.5 \times 10^7 \times e^{-(D-1)/2}\). Thus, the shape \(C_5\) of basal secretion rate is an accurate description under evoked secretion as well, with \(C_5 \approx e^{0.5} = 1.65\). Interestingly, the rosette size required for fusion has been claimed to be at least three SNAREs [17], which would lead via the regression above to a theoretical maximal achievable secretion rate of 13 600 vesicles s\(^{-1}\), twice the maximal observed rate of about 6000 vesicles s\(^{-1}\), that corresponds in the regression equation to a rosette composed of five SNAREs. Perhaps, the minimal in vivo rosette size is 4.

The literature of the past 40 years reports on many studies in which secreted granule content has been carefully monitored, under two conditions: basal and evoked secretion. The basal case should conform to the exit distribution described above, and indeed, the authors have documented good correspondence with the above set of equations [5,16]. The evoked case is generally assumed to be the result of sharply increased Ca\(^{2+}\) concentration, inducing the exocytosis of most or all granules in the vicinity of the plasma membrane porosome complex [6–13]. Porosomes are permanent cup-shaped lipoprotein structures at the cell plasma membrane [8–10], with t-SNAREs at its base. Secretory vesicles transiently dock and
fuse at the porosome base via a fusion pore formed by the assembly of t–v-SNARE complexes in a rosette pattern [8–10]. In various secretory cells, the fusion pore may flicker between closed and open states unless it fully expands (permanent, complete fusion) or closes again (transient, kiss-and-run fusion) [8–13]. If this is the case, evoked secreted granule content should follow the stationary distribution. Strong early evidence in this direction was provided by von Schwarzenfeld [18]. Von Schwarzenfeld observed and measured that under increased neurosecretory cell activation, the leading vesicles along the plasma membrane are exocytosed first. Similar morphological work on classical secretory cells documented, in parallel to von Schwarzenfeld, that granules along the plasma membrane are secreted first, followed by fusion of adjacent inner granules, forming a degranulation sac. This fusion-related membrane advancement towards neighbouring granules by progressive burrows has been termed compound exocytosis [19]. These steps correlate mainly with Ca\(^{2+}\) flow into the cell upon activation.

Basal and evoked secretion states are exocytosis modes of the same pool of granules located in close proximity to the plasma membrane. Secreted granule content should display a distribution that is a mixture of the two, ranging from the exit distribution at the low Ca\(^{2+}\) concentrations typical of basal secretion, to the stationary distribution, at the opposite end of high Ca\(^{2+}\) concentration. The exit distribution EXIT depends [5,16] on the two parameters \(\gamma - \beta\) and \(\mu/\lambda\), and the stationary distribution is given by STAT(\(n\) = constant) that applies simultaneously to fit between the membranes [22]. It was hypothesized that the two modes could be that in the basal mode a successful SNARE rosette releases a single granule (and as such, behaving according to the EXIT distribution), whereas in the evoked mode, once the rosette is effective, a number of granules (with typical stationary behaviour) are released via the progressive burrows of compound exocytosis, all 'hitch-hiking' on the original rosette. Thus, we expect evoked secretion rates to decrease exponentially in the rosette size \(K\), as modelled by the \(M–K\) rate relation. The regression line in figure 1 estimates the decay coefficient \(c_3\) to be 1.65.

The authors have paradigmatically identified [5] the two secretion content distributions with the exit and stationary distributions, and applied maximum-likelihood estimation to 12 examples of published data, to estimate in each case the parameter triple (\(\nu, \sigma\) and \(\tau\)) that applies simultaneously to the two empirical distributions, as well as the quantal skeleton (mixture weights at the various peaks) corresponding to each of the two [5,14,16]. The observation that \(\beta\) and \(\gamma\) have values in the range \(-2\) to \(-6\) led us to abandon attempts at modelling growth and secretion rates by classical physics such as van der Waals forces, in favour of particle physics and statistical mechanics [5], taking SNARE proteins and organelles to centre stage [20–24].

2.2. The \(M–K\) rate event
It has not eluded note that calcium ion is also a significant parameter; after all, it has been documented for more than half a century [4] that secretion rate is proportional to [Ca\(^m\)], where [Ca\(^m\)] is Ca\(^{2+}\) concentration and \(M\) is the number of cooperating ions (0 \(\leq M \leq 5\)), reflecting that multiple Ca\(^{2+}\) ions binding to \(M\)-sites are required for fusion acceleration [2]. Such relation, well documented for exocytosis and recently confirmed also for homotypic fusion [25], is not surprising; Ca\(^{2+}\) ions are the key ions required for the finalization of membrane fusion. In the case of Ca\(^{2+}\) ion binding, the first Ca\(^{2+}\) ion has three to five different locations (depending on the ligand protein) where it can bind [2,8–13]. In case \(M > 1\), the reaction is positively cooperative, namely the first Ca\(^{2+}\) ion binding to a site increases the protein–Ca\(^{2+}\) ion complex affinity at other binding sites. This represents a state of higher entropy compared with binding the last Ca\(^{2+}\) ion, which has only one location where it can bind. Thus, in going from the unbound to the bound state, the first Ca\(^{2+}\) ion must rise above a larger entropy change than the last Ca\(^{2+}\) ion. In an attempt to represent a broader range of secretion rates, the number \(X\) of cooperating Ca\(^{2+}\) ions should be considered random, with \(M\) as an upper bound. It is natural to think of \(X\), the number of ions located in a small vicinity of the binding protein, as being Poisson-distributed [8,9] with some mean \(\eta\). From this point of view, secretion rate should be considered to follow the \(M–K\) rate relation, whose \(M\)-dependence component is rate \(= \frac{{C_1 E_2 \left( {\frac{1}{2} [Ca]^{[M-2]}X,M} \right)}}{X,M}\). The parameter \(C_2\), which adjusts for the units in which [Ca\(^m\)] is measured, can be embedded in \(C_1\) only when the exponent \(M\) is constant. Assuming a rosette diameter of 6 nm and an effective distance between the two membranes [9] of 0.5 nm (5 Å), the volume of the emerging cylinder is \(1.4 \times 10^{-20}\) cm\(^3\). Thus, one Ca\(^{2+}\) ion, that will be caged within such narrow volume, will create a local concentration of 8 mM which is about four magnitudes of the cytoplasmic Ca\(^{2+}\) ion concentration (30 nM) and close to normal blood-ionized calcium level \((\approx 1.9–2.7\) mM\). These significant concentration gradients facilitate a very rapid increase in cytosolic calcium concentration upon opening of calcium channels. Accordingly, there are two options for cytoplasmic Ca\(^{2+}\) ions to diffuse into the effective fusion zone, either individually or bound to a calcium-binding protein (e.g. synaptotagmin). Both options have similar stochastic nature [11].

X-ray diffraction measurements demonstrated that v- and t-SNAREs in opposing membranes overcome repulsive forces, bringing them closer, so that the inter-bilayer space between them is just 2.8 Å [22]. It was hypothesized that hydrated calcium ions (having a shell of six water molecules and measuring nearly 7 Å) could bridge the oxygen (and coordinated water molecules) of the opposing phospholipid head groups, resulting in loss of water from the site, leading to lipid mixing and fusion. It has been demonstrated that if t-SNARE and v-SNARE vesicles are mixed prior to the addition of calcium, the 2.8 Å gap between opposing membranes established by SNAREs would hinder the hydrated calcium measuring nearly 7 Å to fit between the membranes and bridge the opposing phospholipid head groups, preventing membrane fusion [23,24]. This, indeed, turned out to be correct. The findings have further been confirmed by blind molecular dynamics simulations using calcium, dimethylphosphosphate (smallest lipid) and water molecules, demonstrating the bridging of the opposing oxygen atoms of dimethylphosphate and loss of water from the bridged site. The distance between the opposed oxygen atoms bridged by calcium is 2.92 Å, close to the 2.8 Å determined from X-ray measurements. Therefore,
the participation of calcium-binding proteins such as synaptotagmin provides in cells further regulation to the membrane fusion process. This is consistent with the role of the higher local membrane curvature and higher accumulated energy during fusion pore expansion for smaller vesicles.

We propose now to relate the global SNARE particle protein dynamics that differentiate between cell types with the local Ca^{2+} ion cooperation dynamics, to present a unified theory of secretion rate, under which secretion rate is representable as the product of two contributions: the local ion cooperation effect \( C_1(C_2[Ca])^{\text{min}(XM)} \) and the global SNARE effect \( C_\text{SNARE} \). The unified representation for secretion rate is then the \( M–K \) rate relation \( \approx C_1E/C_2[Ca]^{\text{min}(XM)}C_\text{SNARE}^{-K} \).

Even in the absence of secretory cell activation (e.g. action potentials), most cells basally secrete individual granules [1–3,6]. The contribution of Ca^{2+} ion for such events has been least investigated \emph{in vivo}, although, as has been documented [20–26], Ca^{2+} ion was observed in liposome experiments to be mandatory for the fusion process, but not evidently in a cooperative manner (namely \( M = 1 \) is not excluded) [7,20,21,25]. This issue can be investigated by means of statistical analysis based on the G&E theoretical model, via the estimation of the \textit{effective kinetics factor} \((\mu/A)\), the relative rate of granule membrane fusion to granule homotypic fusion. Electronic supplementary material, figures S1 and S2, supports the Ca^{2+} ion-dependent mechanism. It is hard to think of any other ligand-associated molecule that can modify the fusion process along two to three orders of magnitude. Taken together, secretion rate, calcium concentration and rosette size are postulated to satisfy rate \( \approx C_1E/C_2[Ca]^{\text{min}(XM)}C_\text{SNARE}^{-K} \) (with \( C_1 \approx 0.05 \) \( \approx 1.65 \)), the parsimonious relation that holds through all activation states. Figure 1 (and electronic supplementary material, figure S2) exhibits separate evidence for the \( M \) and \( K \) effects, and we are unaware of data in support of their joint effect. The cell should adapt \( M \) and \( K \) as dictated by the activation process, a subject for future research.

### 3. Discussion

As indicated above, in some cells, there is more than one type of secretory granule. The different classes of granules are mobilized in a hierarchy, seemingly adjusted to the different roles these granule types play. Because, in all monitored cases, the granules appear indiscriminately dispersed throughout the cytosol in the cell, this hierarchy must rely on mechanisms that discriminate between the different granule subsets. We suggest that rosette size aggregation probability may dictate the hierarchy in tandem with calcium requirements (\( M–K \) rate relation). Elevation of intracellular Ca^{2+} is known to evoke exocytosis of storage granules, but the (hitherto unidentified) complete molecular machinery by which this occurs is the subject of abundant research. We cannot foresee a simpler mechanism in which calcium may invoke secretion hierarchy in the absence of cooperation with SNARE rosette size. Indeed, Neher & Sakaba [2] documented such a relation. These authors found that when calcium ion concentration is elevated directly at the calyx of Held presynaptic terminal with the use of caged calcium, cleavage of SNAP-25 by botulinum toxin A produces a strong reduction in the calcium sensitivity for release [26]. Thus, fast and slow components of neurotransmitter release can be distinguished (see electronic supplementary material, figure S3). The \( M–K \) rate relation can shed insight on rosette size, estimating it to be about six to eight SNARE units. Because the mean secretory vesicle diameter is 46 nm, the estimated number of SNAREs is 6 \((K = 0.9 \times 46^{0.5} \approx 6.1)\), in good agreement with experimental data. Fluorescent proteins can be genetically fused to target proteins (e.g. SNARE proteins) and consequently offer direct access to specific and stoichiometric aggregation. Stochastic optical reconstruction microscopy analysis combined with quantitative clustering algorithms showed clustering of syntaxin and SNAP-25 molecules [27]. In addition, the data show that small clusters can merge with other clusters, to form super-clusters (above 60 SNAREs). Our model suggests that the small clusters might serve as the basic units to form the rosette.

### 3.1. Granule size affects exit rate

The \( M–K \) statistical mechanics-based equation views the SNARE units as an integer number of undifferentiated particles. Secretion diversity mechanisms in a variety of cell types depend on the molecular subfamily heterogeneity of SNARE proteins. Different combinations of SNARE complexes may constitute the rosette for a given vesicle. The diversity of (K) SNARE regulatory proteins and of (min(X,M)) Ca^{2+} sensor sites on various proteins can induce and regulate fusion. Such diversity may explain the rate variability observed in the endocrine cells, with granule diameter range \( (150 < D < 250 \text{ nm}) \). The rate ratio for such range \((e^5 \approx 150)\) is equal to a calcium concentration factor increase of 5–12 \((M = 3 \text{ or } 2)\, \text{ respectively}\). This model describes the critical impact of SNARE rosette size and local Ca^{2+} concentration on secretion rate. The \( M–K \) relation suggests that vesicle priming is the key step for secretion. It has been documented that primed granules fuse immediately as a response to increase in cytoplasmic Ca^{2+} level (mainly along the plasma membrane). This fusion event is mediated directly by the SNAREs and driven by the energy provided from SNARE assembly. The calcium-sensing trigger for this event is the calcium-binding protein (e.g. calcium sensor synaptotagmin). Granule docking is pre-stage to priming. The SNARE proteins do not appear to be involved in the docking step of the granule lifetime cycle [12,13]. Sensitivity towards intracellular Ca^{2+} as a signal to elicit organization and mobilization has been observed to vary among the different granule subsets, in agreement with the hierarchy of their mobilization [28]. Polymorphonuclear cells rapidly secrete their content, inducing non-specific tissue destruction. As has been well recognized, major distinctions happen between the three different neutrophil granule subsets regarding the extent to which these are mobilized both \emph{in vitro} and \emph{in vivo}: gelatinase positive granules (major axis = 209 nm; minor axis = 85 nm) are secreted more readily than specific lactoferrin positive granules (major axis = 305 nm; minor axis = 124 nm), which again are exocytosed more readily than azurophil myeloperoxidase positive granules \((D > 400 \text{ nm})\) [28–30]. Proteases from azurophil granules may activate the cathelicidin present in the specific granules by proteolytically removing the inhibitory (and protective) N-terminal cathelin-like part of the protein. Similarly, gelatinase and collagenases may be converted from theirzymogen to active proteases by elastase liberated from azurophil granules [28–30]. As such, the small granules containing the substrates are secreted first, whereas the activating enzymes (stored within the large granule) are secreted last. As indicated in previous works [5,16], smaller granules have higher...
probability of basal release. To decrease basal secretion of harmful proteins, the neutrophils terminate granule synthesis (increase A and thus decrease $\mu$/A) and generate oblate granules (and thus increase K).

Hatakeyama et al. [31] investigated exocytosis in pancreatic β-cells with two-photon extracellular polar-tracer imaging. These authors detected marked Ca$^{2+}$-dependent exocytosis of small vesicles (SVs) with a mean diameter of 80 nm in addition to exocytosis of some large vesicles (LVs). Exocytosis of SVs occurred with a time constant of 0.3 s, whereas that of LVs showed a time constant of more than 1 s. Furthermore, they applied photolysis of caged cAMP to quantify the speed of cAMP action during high-glucose stimulation, and found that the augmentation of exocytosis by cAMP occurred within a fraction of a second for SVs but with a delay of 5 s for LVs. Pheochromocytoma PC12 cells provide a definitive example of such a mechanism. In these cells, acetylcholine-containing small clear vesicles (SVs) and monoamine-containing large dense-core vesicles (LVs) undergo exocytosis with time constants of about 30 ms and 10 s, respectively [30–33]. In spite of the specificity of their cargoes and of their intracellular pathways, these secondary vesicles share similar membrane components, and their exocytosis is mediated by the same SNARE system.

References