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Multiple kinetic components and the Ca^{2+} requirements of exocytosis

Haruo Kasai[1*] **and Noriko Takahashi**[2]

[1]*Department of Physiology, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan*
[2]*Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan*

The use of caged-Ca^{2+} compounds to stimulate Ca^{2+}-dependent exocytosis has substantially increased our understanding of this complex process. By this approach, the existence of multiple kinetic components of exocytosis has been established. These components may correspond to a series of sequential steps that lead to a single fusion-ready state (sequential mechanism) or, alternatively, to heterogeneity in secretory vesicles or in fusion-ready states (parallel mechanism). It is suggested that both of these mechanisms can underlie exocytosis of a single type of vesicle (mixed sequential–parallel mechanism). Studies with caged-Ca^{2+} compounds have also indicated that the Ca^{2+} requirement for exocytosis is substantially greater than that suggested by conventional methodologies. This discrepancy is mainly attributable to the underestimation, by imaging studies with high-affinity Ca^{2+} indicators (due to dye saturation), of the local increases in cytosolic Ca^{2+} concentration that trigger the exocytosis of individual vesicles. The effects of local saturation of such indicators are explored by means of a simple theory.

Keywords: caged-Ca^{2+} compounds; PC12 cell; β-cell; acinar cell; secretion

1. INTRODUCTION

Neurons and other secretory cells possess distinct pools of secretory vesicles. The exocytosis of vesicles from the different pools is regulated by local changes in the intracellular environment, most notably by increases in the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_i$). The intricate spatio-temporal organization of Ca^{2+}-dependent exocytosis has presented an obstacle to the elucidation of the molecular and cellular bases of this important process. However, the recent application of caged-Ca^{2+} compounds, which makes it possible to generate instantaneous increases in $[Ca^{2+}]_i$ in a spatially homogeneous manner, has revealed many aspects of the fine organization of Ca^{2+}-dependent exocytosis (Kaplan & Ellis-Davies 1988; Neher & Zucker 1993; Thomas *et al*. 1993; Ellis-Davies & Kaplan 1994; Parsons *et al*. 1995; Oberhauser *et al*. 1996; Ellis-Davies 1998). We will provide brief reviews of two aspects of this organization: multiple pools of vesicles and the Ca^{2+} dependence of their exocytosis (Kasai *et al*. 1996; Ninomiya *et al*. 1996, 1997; Ito *et al*. 1997; Takahashi *et al*. 1997, 1999; Kasai 1999).

2. MULTIPLE KINETIC COMPONENTS OF EXOCYTOSIS

The introduction of caged-Ca^{2+} compounds in the quantification of Ca^{2+}-dependent exocytosis has revealed the existence of multiple kinetic components, the clarification of which should increase our understanding of the multiple chemical reactions that underlie this process (Neher & Zucker 1993; Thomas *et al*. 1993; Parsons *et al*. 1995; Gillis *et al*. 1996; Kasai 1999). Multiple exocytotic components have also been detected during physiological stimulation of secretory cells; however, in such instances, the kinetics of the changes in the local $[Ca^{2+}]_i$ surrounding the secretory vesicles also contribute to the time-course of exocytosis. The homogeneous and instantaneous increases in $[Ca^{2+}]_i$ achieved by caged-Ca^{2+} compounds allow direct examination of the exocytotic reactions downstream of Ca^{2+} binding to putative Ca^{2+} sensors. Exocytosis has been monitored by measuring either membrane capacitance or quantal secretory events; both approaches have associated advantages and disadvantages (Neher 1998). Available data indicate that both sequential (figure 1*a*) and parallel (figure 1*b*) mechanisms underlie exocytotic components (Kasai 1999). We shall also propose a unifying mixed sequential–parallel mechanism (figure 1*d*).

The sequential mechanism assumes the existence of a single type of vesicle with one final fusion step (figure 1*a*). It is the simplest and most appealing mechanism (Neher & Zucker 1993; Parsons *et al*. 1995; Gillis *et al*. 1996) in that it may be possible to directly correlate the exocytotic components with the various molecules that mediate this process (Augustine *et al*. 1996; Calakos & Scheller 1996). The assumption of one final fusion step is consistent with the homogeneous appearance of the vesicle population and the apparently shared fates of the individual vesicles in certain cell types. Several regulated steps that contribute to such a sequential mechanism have been identified: (i) the readily releasable pool of vesicles for Ca^{2+}-dependent exocytosis is augmented by previous increases in $[Ca^{2+}]_i$ (von Rueden & Neher 1993) and by protein kinase C (Gillis *et al*. 1996) in adrenal chromaffin cells; (ii) phosphorylation by cAMP-dependent protein kinase

*Author for correspondence (hkasai@m.u-tokyo.ac.jp).

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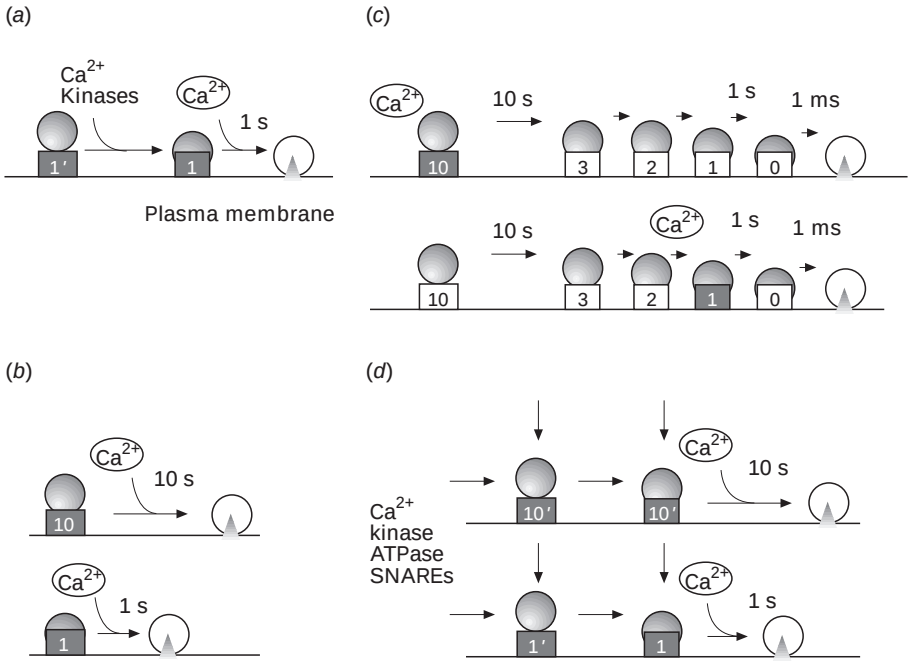

Figure 1. Sequential, parallel and mixed sequential–parallel mechanisms of exocytosis of secretory vesicles. (*a*) The sequential mechanism assumes the existence of only one fusion-ready state (state 1) for Ca^{2+}-dependent exocytosis. Circles represent secretory vesicles, and rectangles the fusion machinery. Ca^{2+} surrounded by an oval indicates the final Ca^{2+}-dependent step. Times indicate approximate time constants for the indicated steps. The readily releasable pool (state 1) of vesicles can be filled either constitutively or in a regulated manner (such as one dependent on the action of Ca^{2+} or protein kinases) from a reserve pool (state 1′). (*b*) The parallel mechanism postulates the existence of more than one fusion-ready state (states 10 and 1) either for vesicles of different types or for vesicles of the same type. (*c*) Preferential filling of sequential states (shaded rectangles) in sequential kinetic steps (the decreasing numbers indicating increasing readiness for fusion) may explain the observed diversity in the time constants of exocytosis. The existence of two sequential pathways with distinct filling states, however, means that the corresponding rate constants are not identical. Such a mechanism can therefore be considered as a variation of the parallel mechanism. (*d*) The mixed sequential–parallel mechanism assumes that the parallel and sequential mechanisms can occur concomitantly, and that one fusion-ready state can undergo a transition into another one. The transition might involve the action of Ca^{2+}, kinase, ATPase or SNAREs. States 1′ and 10′ represent reserve pools for states 1 and 10, respectively.

is necessary for the final triggering of Ca^{2+}-dependent exocytosis in pancreatic β-cells (Takahashi *et al*. 1999); the observation that the ATPase-resistant analogue, ATP-γ-S, could substitute for ATP in this action indicates that such phosphorylation regulates exocytosis at a step downstream of MgATP-dependent vesicle transportation and priming.

In contrast, the parallel mechanism assumes distinct populations of vesicles with fusion-ready states that undergo exocytosis with characteristic time constants (figure 1*b*). 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 (Ninomiya *et al*. 1997; Kasai 1999). This difference is not due to different increases in $[Ca^{2+}]_i$ experienced by the two types of vesicles, because spatially homogeneous Ca^{2+} jumps were applied to the same cells with the use of a caged-Ca^{2+} compound. Similar differences in the time constants for exocytosis between SVs and LVs are also apparent in other secretory cells (Kasai 1999). These time constants widely vary among both vesicle and cell types (Kasai 1999), with predicted values ranging from 0.3 ms for synaptic vesicles, for example, to >10 s for LVs in PC12 cells.

We have also shown that exocytosis of the same type of vesicle, serotonin-loaded LVs, occurs in two phases (mode 1 and mode 2) in pancreatic β-cells (Takahashi *et al*. 1997, 1999). The slow component (mode 2) of LV exocytosis was often selectively recorded in a certain population of cells (Takahashi *et al*. 1997), suggesting that the two modes reflect exocytosis of LVs at distinct fusion-ready states (figure 1*b*). If we interpret the same data with a sequential mechanism, we need to assume two sequential pathways with different filling states of pools (figure 1*c*). Such an assumption implies that the rate constants for the two sequential pathways would differ. Thus, the mechanism can be considered a variant of the parallel mechanism shown in figure 1*b*. Our observation that cytosolic MgATP and phosphorylation selectively augment mode-1 exocytosis, leaving mode-2 exocytosis unaltered (Takahashi *et al*. 1999), provides further support for the existence of two distinct fusion-ready states.

The existence of multiple fusion-ready states for a single type of vesicle is also suggested by the fact that the time constants of fusion for LVs markedly differ between adrenal chromaffin cells and PC12 cells, even though similarly docked LVs are present in both types of cells (Martin & Kowalchyk 1997). In addition, the time constants of exocytosis exhibit substantial cell-to-cell variability (Neher & Zucker 1993; Heinemann *et al*. 1994),

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suggesting that the fusion-ready states may vary depending on the metabolic state of cells. Heterogeneity in the distance between vesicles and the plasma membrane (Plattner *et al*. 1997) as well as in the molecules that mediate exocytosis is also apparent. As an example of the latter diversity, two isoforms of synaptobrevins and of SNAP-25 as well as six isoforms of syntaxins have been identified (Calakos & Scheller 1996). It is possible that fusion rates are determined by the particular isoforms of such proteins and their local concentrations at the site of fusion; thus, membrane fusion mediated by influenza virus haemagglutinin requires multiple haemagglutinin trimers (Daniele *et al*. 1996). Many putative Ca^{2+} sensors have also been identified, including nine forms of synaptotagmin, rabphilin3A, Doc2, Munc13, and CAPS (Calakos & Scheller 1996). Thus, it is not realistic to assume that the identical fusion machinery underlies the diverse fusion kinetics of Ca^{2+}-dependent exocytosis for all cell and vesicle types. It seems more likely that different configurations of fusion machinery or morphological relations are responsible for the observed kinetic diversity.

It is therefore conceivable that a single type of vesicle can undergo exocytosis by both sequential and parallel mechanisms. In the mixed sequential–parallel mechanism depicted in figure 1*d*, a single type of vesicle has more than one fusion-ready state, and transitions between such states are possible. These transitions might not be as rapid as the final fusion events, and they might not substantially contribute to the multiple components of exocytosis detected within a short period of experimental observation (1–6 min). It seems appropriate to consider this type of model for further clarification of the molecular basis of Ca^{2+}-dependent exocytosis, because neither pure-sequential nor pure-parallel models can fully account for the available data. Indeed, a similar idea has been proposed to explain the observed diversity in capacitance measurements in chromaffin cells (Heinemann *et al*. 1994). Experimental validation of these models must include the identification of the molecular processes that determine the diverse time constants of exocytosis.

3. MEASUREMENT OF LOCAL $[Ca^{2+}]_i$

Before the introduction of caged-Ca^{2+} compounds, the physiological increases in $[Ca^{2+}]_i$ required to trigger exocytosis of fusion-ready vesicles were thought to be less than 1 μM. This assumption was based on measurements of the increases in $[Ca^{2+}]_i$ during exocytosis with the use of high-affinity indicator dyes such as fura-2, which yielded values of less than 1 μM in most secretory cells examined. For example, such increases were estimated to be less than 0.1 μM in presynaptic nerve terminals (Smith *et al*. 1993) and 1 μM in chromaffin cells (Cheek *et al*. 1989), pancreatic β-cells (Pralong *et al*. 1988; Bokvist *et al*. 1995), and pancreatic acinar cells (Kasai & Augustine 1990; Toescu *et al*. 1992). Although exocytosis in permeabilized preparations often required relatively large half-maximal Ca^{2+} concentrations, such as 3 μM in adrenal chromaffin cells (Burgoyne 1991) and 10 μM in PC12 cells (Ahnert-Hilger *et al*. 1987), pancreatic β-cells (Okazaki *et al*. 1994), and pancreatic acinar cells (Muallem *et al*. 1995), often these values were not considered physiological (Walent *et al*. 1992). One exception was exocytosis of synaptic vesicles, for which the possibility of a high local $[Ca^{2+}]_i$ regulating exocytosis had been proposed even in early studies, because of the theoretical considerations that are required to explain the rapid termination of Ca^{2+}-dependent exocytosis during neurotransmitter release (Simon & Llinás 1985; Zucker & Fogelson 1986).

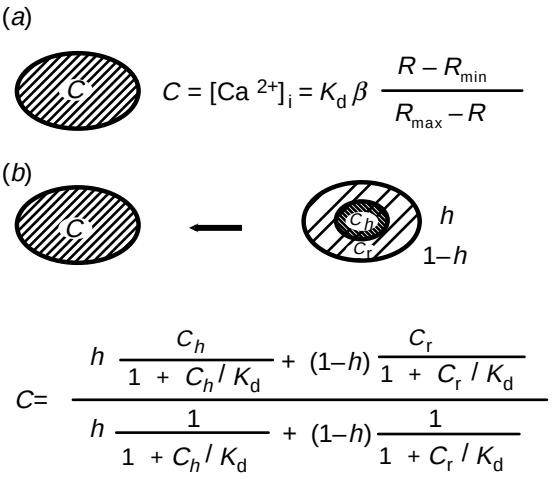

Figure 2. Ratiometric measurement of $[Ca^{2+}]_i$. (*a*) The $[Ca^{2+}]_i$, or *C*, can be precisely calculated from ratiometric measurements provided that it is homogeneous in the region of measurement and that the calibration constants—the minimal (R_{min}) and maximal (R_{max}) fluorescence ratios, the dissociation constant (K_d), and β (Grynkiewicz *et al*. 1985)—are precisely determined. (*b*) The estimated $[Ca^{2+}]_i$, or *C*, is smaller than the mean value in the area of measurement when there is heterogeneity in Ca^{2+} concentration. This is because of saturation of the Ca^{2+} indicator dye in regions of high $[Ca^{2+}]_i$ (Ca^{2+} hot spots) within the pixel. Assuming that the $[Ca^{2+}]_i$ is C_h in the hot spot and C_r in the rest of the pixel, and that the volume fraction of the hot spot within the pixel is *h*, the estimated value (*C*) of $[Ca^{2+}]_i$ is provided by a weighted mean of C_h and C_r as indicated.

However, photolysis of caged-Ca^{2+} compounds in cells in the whole-cell patch-clamp configuration and measurement of $[Ca^{2+}]_i$ with low-affinity Ca^{2+} indicators indicated that the half-maximal Ca^{2+} concentration for the exocytosis of fusion-ready vesicles was 200 μM in presynaptic terminals (Heidelberger *et al*. 1994), 30 μM in pituitary melanotrophs (Thomas *et al*. 1993), and 20 μM in adrenal chromaffin cells (Chow *et al*. 1994; Heinemann *et al*. 1994), pancreatic β-cells (Takahashi *et al*. 1997), and pancreatic acinar cells (Ito *et al*. 1997). Thus, the low affinity of the final Ca^{2+}-dependent step in exocytosis has been demonstrated consistently in permeabilized preparations and in caged-Ca^{2+} experiments, both situations in which the increase in $[Ca^{2+}]_i$ is applied homogeneously.

The discrepancy in the Ca^{2+} requirements of exocytosis as determined by physiological stimulation and with the use of caged-Ca^{2+} compounds is thought to result largely from local saturation of the Ca^{2+} indicator dye in the former situation. A simple and quantitative explanation of such local saturation can be described as follows: if the gradient of $[Ca^{2+}]_i$ is fully resolved, meaning that there is no spatial gradient within one pixel of an image, then

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$[Ca^{2+}]_i$ should be precisely obtained from the fluorescence ratio (R) of the indicator dye according to the equation of Grynkiewicz *et al.* (1985) (figure 2*a*). This is not the case, however, if there is an unresolvable gradient of $[Ca^{2+}]_i$ within a pixel along the *x*-, *y*-, or *z*-axis or in the time dimension. Let us assume that $[Ca^{2+}]_i$ is high (C_h) in a small volume fraction h within a pixel and that it is C_r elsewhere within the pixel. The estimated $[Ca^{2+}]_i$ would then be the spatial average, $h\,C_h+(1-h)C_r$, if C_h is small and R responds linearly to $[Ca^{2+}]_i$. However, if C_h is not small and the effect of saturation is not negligible, the estimated $[Ca^{2+}]_i$ is the weighted mean value as shown in figure 2*b*; in this case, the more C_h increases the less it contributes to the mean value. Consequently, if the dye is already saturated at C_h, any further increase in C_h has a negligible effect on the weighted mean value. Thus, estimation of $[Ca^{2+}]_i$ is limited by the affinity of the Ca^{2+} indicator dye used, and $[Ca^{2+}]_i$ is underestimated. The presence of such local saturation does not result in overall saturation of R, which can be further increased by increases in the volume (h) of the hot spot. Therefore, transient, local, and large increases in $[Ca^{2+}]_i$ can easily be missed if unresolvable spatial $[Ca^{2+}]_i$ gradients exist.

We have experienced an example of the local saturation of fura-2 in pancreatic acinar cells (Ito *et al.* 1997). Estimations of agonist-induced increases in $[Ca^{2+}]_i$ based on fura-2 measurements have usually yielded peak values of less than 1 μM, as the values at which agonist-induced increases in membrane capacitance, reflecting exocytosis, are apparent. However, our experiments with a caged-Ca^{2+} compound indicated that an increase in $[Ca^{2+}]_i$ of more than 5 μM was necessary for an increase in membrane capacitance. We therefore monitored $[Ca^{2+}]_i$ with a low-affinity Ca^{2+} indicator, BTC, and found that the estimated increase in $[Ca^{2+}]_i$ can exceed 10 μM, especially at the secretory pole of the cells. As expected from the theory described above, fura-2 gave a lower value of $[Ca^{2+}]_i$ at the trigger zone, where Ca^{2+} hot spots are clustered. The Ca^{2+} waves demonstrated with the low-affinity Ca^{2+} indicator were more transient, more localized, and larger than those measured with the high-affinity indicator. All of these differences between the Ca^{2+} images obtained with a low-affinity dye and those obtained with a high-affinity dye can be explained by the local saturation of the latter, as indicated above. Thus, imaging with the low-affinity Ca^{2+} indicator provided an estimate of the Ca^{2+} requirement of exocytosis consistent with that predicted from the use of caged Ca^{2+} in exocrine acinar cells.

There are two additional advantages to the use of low-affinity indicators for the realistic estimation of $[Ca^{2+}]_i$. First, all the calibration constants of indicator dyes must be obtained *in vivo*, because they depend on the cytosolic environment (Roe *et al.* 1990). The precise determination of the maximal R value (R_{max}) is especially problematic because $[Ca^{2+}]_i$ must be increased to a sufficiently high value for a certain period of time, possibly with a substantial impact on the cytosolic environment. The R_{max} values thus estimated show marked cell-to-cell variation, and they cannot always be very precise. Consequently, as R approaches R_{max}, estimation of $[Ca^{2+}]_i$ becomes less reliable, as can be seen from the equation in figure 2*a*. This problem is less significant with low-affinity Ca^{2+} indicators than with high-affinity indicators because R is less likely to achieve values close to R_{max}. The second additional advantage of low-affinity Ca^{2+} indicators is that, unlike high-affinity indicators, they do not substantially affect Ca^{2+} signalling; the buffering effect of high-affinity indicators can markedly slow the kinetics of such signalling, resulting in the underestimation of Ca^{2+} requirements if secretion and Ca^{2+} measurements are made in different preparations.

The use of low-affinity Ca^{2+} indicators is thus recommended, from many standpoints, for the quantitative investigation of Ca^{2+} signalling. The combination of such indicators with tomographic imaging methods should yield even more precise estimations of the Ca^{2+} dependence of exocytosis during physiological stimulation. To achieve this goal, however, we need to cope with the small signals generated by the limited number of available low-affinity Ca^{2+} indicators or to develop new, more efficient dyes.

4. CONCLUSIONS

The use of caged-Ca^{2+} compounds has revealed that the Ca^{2+} requirement of exocytosis tends to be higher than estimated by Ca^{2+} imaging methods with high-affinity Ca^{2+} indicators, and that there is heterogeneity in the fusion-ready states of secretory vesicles. However, sustained experimental efforts are required not only to quantify the molecular steps of exocytosis, but also to achieve consistency in the results obtained with different preparations and by different laboratories.

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