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Ca^{2+}-Dependent Exocytosis in Endocine, Exocrine, and Nonsecretory Cells

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Exocytosis in various secretory cells is regulated by Ca^{2+} signaling. In this minireview, I will introduce our recent approach, which we have termed comparative biology of exocytosis, to the study of Ca^{2+}-dependent secretion in such cells. In this approach, we quantify and compare the secretory process in different cell types (neurons, endocrine cells, and exocrine cells) with the same techniques. This approach benefits from the fact that the biochemistry and ultrastructure of these cells are relatively well characterized and it is expected to be particularly revealing because of the marked differences in the properties of exocytosis thought to exist among different secretory cells. The first part of this article deals with the mechanism by which Ca^{2+} signaling regulates exocytosis in exocrine cells, and the second part deals more generally with the diversity in the kinetics of the exocytotic machinery among different types of cells and secretory vesicles.

Ca^{2+} signaling in exocrine cells

I will first introduce some basic features of Ca^{2+} signaling in pancreatic acinar cells. When enzymatically dissociated pancreatic acinar cells are loaded with a Ca^{2+} indicator such as fura-2 and stimulated with aceycholine (ACh) (1 to 10 μM) or cholecystokinin (CCK), the resulting increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$)always begins in a small region of the apical (luminal) portion of the cell, known as the trigger zone, and then spreads to the basal region, which normally faces the bloodstream and in the plasma membrane of which aponist receptors are distributed. These Ca^{2+} waves are not acutely dependent on external Ca^{2+}, and are mediated by Ca^{2+} release from intracellular stores. In response to lower concentrations of ACh (50 to 100 nM), the Ca^{2+} spike occurs locally within the apical region of the cell (L-spike, Fig. 1) and exhibits oscillations.

The mechanisms underlying the Ca^{2+} waves have been investigated by injecting inositol 1, 4, 5-trisphosphate (IP_3), a putative second messenger of ACh that induces the release of Ca^{2+} from intracellular stores, into the basal region of the cell with the use of a patch pipette(Kasai et al, 1993). Rupture of the patch membrane immediately results in a small increase in

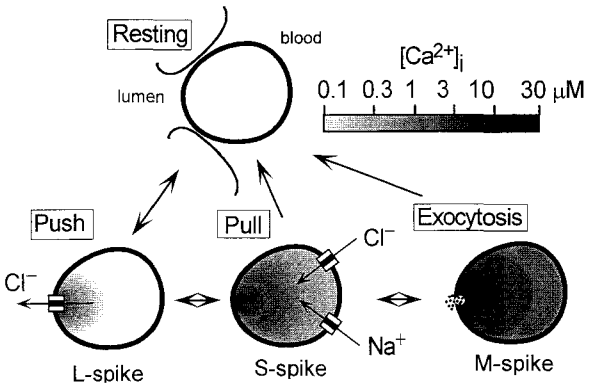

Fig. 1. Three active phases of acinar cell secretion. A local Ca^{2+} spike (L-spike) at the trigger zone induces secretion of Cl^- into the lumen of an acinus (push phase). A global submicromolar Ca^{2+} spike (S-spike) sequentially activates luminal and basal ion channels, and sequentially induces the push phase and uptake of Cl^- from the blood (pull phase). A micromolar Ca^{2+} spike (M-spike) induces exocytosis of zymogen granules (exocytosis phase). The occurrence of three active phases of secretion is regulated in an agonist concentration-dependent manner. Modified with permission from Ito et al (1997).

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$[Ca^{2+}]_i$ close to the injection site, indicating that the basal region of the cell expresses IP_3 receptors. This effect is then followed by a marked increase in $[Ca^{2+}]_i$ at the trigger zone, which is located at the opposite side of the cell relative to the injection site. These observations indicate that IP_3 sensitivity is particularly high at the trigger zone and that IP_3 diffuses readily within acinar cells. In contrast, increases in $[Ca^{2+}]_i$ can be confined to a small area, as a result of the action of immobile Ca^{2+} buffers and Ca^{2+} pumps. These fundamental mechanisms enable acinar cells to generate Ca^{2+} waves in a spatially organized manner, irrespective of the localization of agonist receptors (Kasai & Petersen, 1994).

Two functions of exocrine acinar cells are regulated by increases in $[Ca^{2+}]_i$: electrolyte secretion, primarily mediated by the activation of Cl^- channels, and enzyme secretion, mediated by exocytosis of zymogen granules (large, dense-core vesicles, or LVs, of exocrine cells). Both types of secretion ultimately take place at the luminal membrane. Although the regulation of Cl^- channels has been intensively studied (see below), the precise Ca^{2+} and time dependencies of exocytosis have not been systematically investigated under physiological conditions.

To characterize these properties of exocytosis, one cannot rely on agonist stimulation because it generates IP_3, which, in turn, produces such complex increases in $[Ca^{2+}]_i$ that it is not possible to determine the precise Ca^{2+} concentrations around individual zymogen granules. We have therefore used caged-Ca^{2+} compounds, which are Ca^{2+} buffers with a nitrobenzyl side chain. On exposure to ultraviolet (UV) radiation, the tetracarboxyl Ca^{2+} binding site of these compounds is cleaved in two, resulting in an approximately 10,000-fold decrease in Ca^{2+} affinity. Thus, by infusing large concentrations (10 mM, plus 5 mM Ca^{2+}) of these compounds, to overcome endogenous Ca^{2+} buffering, into cells, and then applying a flash of UV radiation, we can create homogeneous, rapid, and large increases in $[Ca^{2+}]_i$, or Ca^{2+} jumps.

We measure membrane capacitance in order to quantify exocytosis. Increases in capacitance of the plasma membrane reflect increases in membrane area caused by fusion of cytoplasmic organelles. Photolysis of caged-Ca^{2+} compounds and measurement of membrane capacitance can be readily combined, because both methods rely on patch clamp methodology. In such experiments, patch pipettes contain both a caged-Ca^{2+} compound and a Ca^{2+} indicator dye. After the solution in the pipette has equilibrated with cytosol, a flash of UV radiation is applied to produce a Ca^{2+} jump, and the $[Ca^{2+}]_i$ is ratiometrically monitored with the Ca^{2+} indicator.

In measuring membrane capacitance, it is important to be aware of the possibility of cross talk from changes in membrane conductance. This complication is especially relevant with exocrine cells because of the abundance of Ca^{2+}-dependent channels that contribute to electrolyte secretion and the lack of selective blockers for such channels. We have avoided this problem by replacing all the internal and external cations with *N*-methyl-D-glutamate and all external and internal anions with glutamate. Under these conditions, Ca^{2+} jumps induce negligible currents, but they result in a slow and delayed increase in capacitance. This increase in capacitance persists for the duration of the period in which the increase in $[Ca^{2+}]_i$ is maintained. We demonstrated that exocytosis was not triggered by a Ca^{2+} jump to 5 μM, whereas slow exocytosis was triggered at a $[Ca^{2+}]_i$ of >6 μM. At 8 μM Ca^{2+}, a small fast component of exocytosis appeared together with the slow component, and, at 20 μM, the fast component of became more pronounced. The Ca^{2+} dependencies of the rates of the fast and slow components of exocytosis indicate that the slow exocytosis is more sensitive to Ca^{2+}. The Ca^{2+} dependence of slow exocytosis was similar to that of amylase secretion from permeabilized acinar cell preparations, indicating that the slow component likely reflects the exocytosis of LVs.

The observation that exocytosis in pancreatic acinar cells, as detected by measurement of capacitance, required a $[Ca^{2+}]_i$ of >5 μM appeared inconsistent with the results of previous physiological studies, none of which had detected such large increases in $[Ca^{2+}]_i$ during agonist stimulation under conditions known to trigger exocytosis, even in cells patch-clamped in the whole-cell mode (Maruyama, 1988). Two possibilities could account for this discrepancy. First, small domains of high $[Ca^{2+}]_i$ might exist around the mouths of the Ca^{2+} release channels that trigger exocytosis. Such regions of high $[Ca^{2+}]_i$ have been called Ca^{2+} domains and are thought to trigger exocytosis of synaptic vesicles. Unlike synaptic transmission, however, we could readily block agonist-induced exocytosis in exocrine cells with exogenous Ca^{2+} buffers, therefore excluding the Ca^{2+} domain hypothesis. Second, large increases in $[Ca^{2+}]_i$ might

not have been detected previously because most studies used high-affinity Ca^{2+} indicators, which can be readily saturated if the $[Ca^{2+}]_i$ increases to >5 μM.

We therefore subjected acinar cells to Ca^{2+} imaging with a low-affinity Ca^{2+} indicator, benzothiazol coumarin (BTC). With this approach, we showed that the increase in $[Ca^{2+}]_i$ induced by ACh (1 μM) transiently exceeded 10 μM. Furthermore, the region of high $[Ca^{2+}]_i$ was restricted to the trigger zone in the secretory area, with the increase in $[Ca^{2+}]_i$ recovering slowly over a period of several seconds. Such a transient and localized Ca^{2+} spike had never been detected in images acquired with fura-2, with which no Ca^{2+} gradient could be seen after the Ca^{2+} wave had spread to the basal area and the $[Ca^{2+}]_i$ increase appeared sustained. The characteristics of the fura-2 images can now be readily explained as resulting from saturation of the dye within small areas in the trigger zone that were not resolved by the method of imaging. This hypothesis was further confirmed with the use of the two Ca^{2+} indicators simultaneously (Ito et al, 1997).

We showed that the micromolar Ca^{2+} spike, or M-spike, could fully account for exocytosis in acinar cells. When the M-spike was induced by agonist, a slow increase in membrane capacitance was detected. However, when Ca^{2+} was buffered so that $[Ca^{2+}]_i$ remained at <5 μM, no capacitance increase was induced by agonist. Thus, the large amplitude and long duration of the M-spike appear adequate for regulating exocytosis in exocrine acinar cells.

Lower concentrations of agonist (50 nM) triggered a different type of Ca^{2+} spike, called the S-spike, that did not exceed 3 μM. During the S-spike, the Ca^{2+} wave can spread from the trigger zone to the basal area, as with the M-spike, and result in a biphasic activation of Ca^{2+}-dependent ion channels. The early Cl^- current was activated as soon as the increase in Ca^{2+} concentration was detected at the trigger zone, indicating that the corresponding Cl^- channels are localized in the luminal membrane. An unusual feature of the early Cl^- current was that it decayed rapidly, even though $[Ca^{2+}]_i$ at the trigger zone was still increasing. This observation suggests that the apical Cl^- channels have intrinsic inactivation mechanisms. In contrast, the activation of the late Cl^- current paralleled changes in the average $[Ca^{2+}]_i$ in the basal area, indicating that the corresponding Cl^- channels are distributed throughout the basal area.

This behavior of Cl^- currents was described by George Augustine and I in 1990 (Kasai & Augustine, 1990), and we proposed a push-pull mechanism for Cl^- secretion, whereby sequential activation of different sets of ion channels can act like a valve to maintain the unidirectional flow of chloride ions. We now know that one additional Ca^{2+} signaling state exists for exocytosis. Thus, we have upgraded the original push-pull model into the one shown in Fig. 1. In this new model, three distinct Ca^{2+} spikes (L-, S-, and M-spikes) are induced in an agonist concentration-dependent manner, with each type of spike triggering specific functions in the cell: The L-spike triggers the push phase of electrolyte secretion, the S-spike further induces the pull phase (electrolyte uptake) of electrolyte secretion, and the M-spike triggers exocytosis. Increasing the agonist concentration increases not only the flow of pancreatic juice but also the concentration of digestive enzymes. Our new theory (Fig. 1) thus provides a simple explanation for these fundamental features of exocrine tissue physiology.

This example of exocrine cells has revealed three general conclusins for cell physiology: (1) The same agonist can trigger different types of Ca^{2+} spikes, which, in turn, induce distinct cellular functions in a concentration-dependent manner. (2) Ca^{2+} spikes can be as large as 10 μM, even in epithelial cells. Moreover, even at such high Ca^{2+} concentrations, Ca^{2+} spikes are spatiotemporally organized in such a manner as to optimize the functioning of the cell. The increase in $[Ca^{2+}]_i$ was highest at the trigger zone, where exocytosis takes place, and it was transient, thereby avoiding toxic effects on the cell. And (3) the elaborate nature of Ca^{2+} signaling is revealed only with the use of low-affinity Ca^{2+} indicators. Such indicators have the additional advantage that they influence the native Ca^{2+} buffering system of cells to a smaller exent than do high-affinity dyes. Thus, it will be of interest to reinvestigate various cell preparations with low-affinity indicators.

Kinetic diversity in exocytosis

In the first section, I have described how a Ca^{2+} spike of relatively long duration, the M-spike, regulates exocytosis in exocrine acinar cells. In presynaptic nerve terminals, however, the presence of voltage-gate Ca^{2+} channels in active zones allows the generation of ultrafast Ca^{2+} spike and secretion re-

sponses. In endocrine cells, the relation between Ca^{2+} channels and secretory vesicles appears to be less strict than in neurons, resulting in slower secretion. Are these differences in Ca^{2+} signaling solely responsible for the differences in the rate of exocytosis in these various cell types, or does the rate of exocytotic events downstream of Ca^{2+} binding to putative Ca^{2+} sensors differ among different secretory cells and vesicles? To my knowledge, this question has never been addressed previously, and our data indicate that there is a large kinetic diversity in the exocytotic machinery among different cells and vesicles. We reached this conclusion by considering an important aspect of secretion: Most secretory cells possess two distinct types of vesicles.

The classical example of the existence of two types of secretory vesicles is provided by presynaptic terminals, which possess both synaptic vesicles, containing the primary neurotransmitter, and large, dense-core vesicles (LVs), containing neuropeptides. Synaptophysin was shown to be a specific marker for synaptic vesicles in neurons, and it was subsequently discovered that all endocrine cells express this protein on their synaptic vesicle-like microvesicles, or SVs. The distinction between SVs and LVs has been most intensively investigated in clonal pheochromocytoma PC12 cells, in which SVs selectively contain ACh and LVs contain monoamines, and both types of vesicles undergo exocytosis in a Ca^{2+}-dependent manner. We therefore decided to investigate the kinetics of exocytosis in PC12 cells, hoping that the kinetics for each type of vesicle could be examined separately.

The increase in membrane capacitance induced by large Ca^{2+} jumps (caused by photolysis of a caged-Ca^{2+} compound) in PC12 cells displayed two components. Both components were often, although not always, followed by a decrease in capacitance, which we interpret as resulting from endocytosis (Kasai et al, 1996). If we assume the existence of only one type of secretory vesicle, then the two components of exocytosis must be attributable to sequential kinetic steps that lead to the final fusion event, as has been proposed by Neher (Neher & Zucker, 1993) and Almers (Thomas et al, 1993) in chromaffin and pituitary cells, respectively. However, if we take into account the existence of two types of vesicles, the same data can be interpreted in an entirely different way: SVs may be responsible for the fast component of the capacitance increase, and LVs for the slow component. Our capacitance measurements provided indirect support for the two-vesicle hypothesis (Kasai et al, 1996).

To substantiate further the two-vesicle hypothesis in PC12 cells, we needed to measure ACh secretion during the fast increase in capacitance and monoamine secretion during the slow component of the capacitance increase. For this purpose, we used skeletal myoballs as a biosensor for ACh (Ninomiya et al, 1997). Fully differentiated myotubes expressing large numbers of nicotinic ACh receptors were prepared and then treated with colchicine to make them round and maleable, and PC12 cells were plated on this muscle preparation immediately before patch clamp experiments.

Although these experiments were difficult, we succeeded in several instances. Ca^{2+} jumps in PC12 cells evoked a rapid increase in membrane capacitance, during which we detected transient inward currents, similar to endplate currents (EPCs), in the skeletal myoballs. These EPC-like currents were never detected in the presence of curare, showing that they were induced by ACh released from PC12 cells. We further believe, for three reasons, that each EPC-like current reflects exocytosis of a single SV containing ACh: First, more than one EPC-like current was detected in response to a single Ca^{2+} jump, which is difficult to explain by fusion of a large number of SVs that contain a small amount of ACh, but is readily understood by assuming fusion of a small number of SVs each containing a large amount of ACh. Although the number of SV fusion events predicted from capacitance measurements was $>$ 1000, whereas at most 10 EPC-like events were recorded, this discrepancy can be explained by the fact that PC12 cells contain only small amounts of ACh. We have examined more than 10 clones of PC12 cells, and obtained positive data from only two, with the best one being separated by about 90 passages from the original PC12 cells established by Greene & Tischler (1976). We therefore believe that only a small portion of SVs contains sufficient ACh to be detected by the myotube bioassay.

Second, spontaneous EPC-like currents were also detected, as is the case with EPCs (Katz, 1969), further supporting our SV hypothesis. And finally, the time courses of the EPC-like currents were slightly variable. Such variability can be predicted from the fact that we plated the PC12 cells onto the differentiated myoballs immediately before experiments, so

that exocytosis can occur at any site on the surface of the PC12 cells. The time course of the EPC-like currents was simulated with a simple diffusion equation (Ninomiya et al, 1997), in which we assume that the muscle cell is an infinite absorbing plane for ACh, and that ACh diffuses from an instantaneous point source at a distance *d* from the muscle plane. With this equation, we can predict the time course of EPC-like currents by adjusting only one parameter, the distance of the release site from the muscle. Most EPC-like currents can be well fitted by this equation, giving a predicted distance of the release site from the muscle plane. The distribution of these distances for >40 EPC-like currents revealed that all of the release sites were closer than 10 μm from the muscle plane, a distance smaller than the diameter of a PC12 cell, consistent with the idea that ACh is released from a point source on the studied PC12 cell.

Thus, the EPC-like currents are thought to reflect exocytosis of SVs in PC12 cells. We could then examine the temporal relation between the EPC-like currents and the fast component of the capacitance increase. The EPC-like currents appeared rapidly after the Ca^{2+} jump. The latency histogram constructed from >40 experiments revealed that 95% of the fusion events occurred within 100 ms after the Ca^{2+} jump. Closer investigation of the histogram revealed that there was a delay of about 20 ms and that the SVs were exhausted with a time constant of about 24 ms. Capacitance increases were measured in the same group of cells. Because the increase in capacitance represents the integral of fusion events, the time course of fusion should be given by the derivative of the capacitance increase with respect to time. The time course of exocytosis determined by this aproach also showed a delay of about 20 ms, and the major time constant for SV exhaustion was about 29 ms, similar to the value obtained by analysis of the EPC-like currents in the muscle cells. The marked similarity in the values of these parameters generated from the two sets of data is surprising given that the data were obtained by entirely different methods: capacitance measurement of the increase in presynaptic membrane area, and the postsynaptic detection of ACh secretion. This convergence of the data supports the vesicle hypothesis of ACh secretion from PC12 cells, and indicates that the fast component of the capacitance increase represents the fusion of SVs containing ACh with a time constant of 25 to 30 ms.

Next, we measured monoamine secretion from PC12 cells with the use of carbon fiber electrodes that can record oxidative currents due to monoamines released from single LVs. Quantal monoamine secretion in response to a Ca^{2+} jump was apparent after a delay of more than several hundred milliseconds. Exocytosis of LVs occurred in a sustained manner, consistent with the slow component of the increase in membrane capacitance; there was clearly no LV exocytosis during the fast increase in capacitance. Thus, both capacitance measurement and amperometry indicated that LV exocytosis occurred in a slow and sustained manner. It was not possible to estimate the time constant of fusion for LV exocytosis, other than to say that it is >10 s, because the process is too slow.

Thus, we have substantiated the two-vesicle hypothesis and further demonstrated a >400-fold difference between the time constants of exocytosis for SVs and LVs in the same type of secretory cell and in response to the same Ca^{2+} jumps. We next investigated whether the time course of LV fusion in other cells is similar to that in PC12 cells. For this purpose, we studied adrenal chromaffin cells with the same approach and experimental conditions. Chromaffin cells secreted markedly greater amounts of monoamines than did PC12 cells, consistent with the fact that chromaffin cells contain far larger amounts of these compounds than do PC12 cells. More importantly, the amperometric events in chromaffin cells occurred markedly faster than did those in PC12 cells, with a time constant for LV exhaustion of about 1 s. As in PC12 cells, the increase in capacitance in chromaffin cells showed two components. The time derivative of the capacitance increase revealed that the slow components paralleled the increase in monoamine secretion, consistent with the idea that this component represents exocytosis of typical LVs containing large amounts of monoamines. In contrast, the fast component of exocytosis was associated with relatively little secretion of oxidative substances. In many instances, we did not observe any quantal events during the fast phase, indicating that this component does not reflect fusion of typical LVs. The simplest and most likely explanation for these observations is that the fast component of the capacitance increase in chromaffin cells represents exocytosis fo SVs, given that chromaffin cells, like PC12 cells, are known to contain many synaptophysin-positive SVs.

In these experiments, we assumed the simplest

model for Ca^{2+}-dependent exocytosis, in which the Ca^{2+} binding step precedes the fusion step. Because we applied large Ca^{2+} jumps, the Ca^{2+} binding step should occur rapidly, so that the time constant for exocytosis should reflect that for vesicle exhaustion and decay of the quantal events. In chromaffin cells, LV fusion occurred rapidly, and hence exhaustion occurred rapidly with a time constant of about 1 s, whereas in PC12 cells exhaustion of LV secretion required >10 s. Thus, the fusion time constant for the same type of vesicle can differ markedly among different cell types.

We have performed a systematic investigation of capacitance changes in various cells including PC12 cells, chromaffin cells, pancreatic β cells, pancreatic acinar cells, hematopoetic RBL cells, and nonsecretory CHO fibroblasts. Each type of cell shows a characteristic time course of exocytosis and endocytosis. However, one feature was shared by all secretory cells: the existence of two components of exocytosis with markedly different time constants. Amperometric measurements indicated that LV exocytosis was associated only with the slow component of exocytosis in PC12 cells and chromaffin cells, as described above, as well as in pancreatic β cell. Incontrast with secretory cells, CHO cells show only one major component of exocytosis. Because they do not contain LVs, the capacitance change in these cells must be mediated by vesicles similar to SVs. Thus, the capacitance data correlate well with the morphological features of the cells investigated.

These analyses have revealed that the time constants for exocytosis of the two types of vesicles in various cells, shown plotted on a logarithmic sclae in Fig. 2, exhibit a marked diversity. Exocytosis is fastest in cells in which the most rapid secretion would be expected in vivo, and is slow in cells, such as exocrine cells, in which secretion should occur slowly. Thus, the time constants of exocytosis appear optimized for cellular functions. The precise functional relevance of the time constant of exocytosis can be understood from the following considerations. If we assume that a large Ca^{2+} spike of duration t is applied and that the time constant of exocytosis, τ, is substantially greater than t, then the probability of fusion is approximated by t/τ. Thus, the probability of fusion is determined by t and τ. Moreover, t and τ should be relatively well balanced for effective control of secretion: If τ is too large relative to t, then little secretion will occur and Ca^{2+}

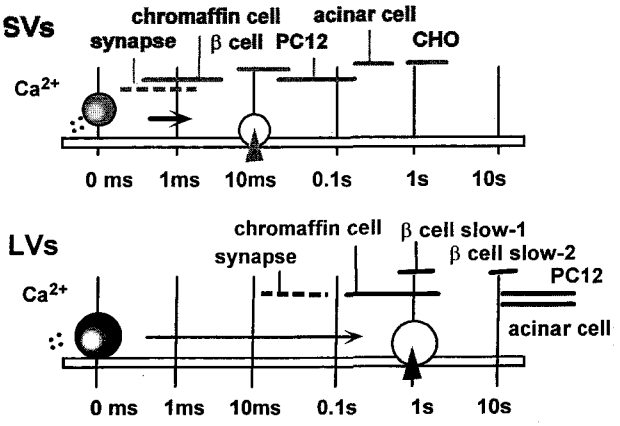

Fig. 2. Diversity in the time constants of exocytosis. Logarithmic plot of the time constants for exocytosis of SVs and LVs in various cell types as determined with the use of caged-Ca^{2+} compounds. Solid bars show the values derived from measurements of quantal secretion for SVs in PC12 cells and LVs in PC12 cells, chromaffin cells, and pancreatic β cells. The values for SVs in chromaffin cells, β cells, acinar cells, and CHO cells and for LVs in acinar cells are based on capacitance measurements. Dotted bars indicate the predicted values for SVs and LVs in presynaptic terminals.

signaling will be wasted; on the other hand, if τ is too small, the pool of secretory vesicles will be readily exhausted. Two extreme examples of such a balance are presynaptic terminals and exocrine cells. In presynatic terminals, both t and τ are thought be <1 ms (Fig. 2), whereas in exocrine cells, where long Ca^{2+} spikes regulate slow exocytosis, both t and τ are on the order of 10 s.

Thus, both Ca^{2+} signaling and exocytosis appear optimized for the functioning of each type of secretory cell. The time constant of exocytosis is expected to be as important a parameter for secretion as is Ca^{2+} signaling. The modification of this time constant in neurons and endocrine cells might be expected to result in synaptic plasticity and clinical disorders, respectively.

The mechanism underlying this diversity in time constants of exocytosis remains largely a mystery, but our approach has shed light on two steps. it has been suggested that there is a final ATP-dependent step in exocytosis, termed the priming step, that is possibly mediated by NSF and the SNARE complex and after which vesicles are ready for the Ca^{2+} trigger. However, most of the exocytosis in our studies was independent of cytosolic ATP, and the time constant of exocytosis should therefore reflect that of the final

reaction. In endocrine cells, primed LVs are thought to be docked to the plasma membrane, so that the diversity in the time constant of exocytosis should reflect differences in the time constant of the fusion reaction after docking. In contrast, LV docking is rarely observed in exocine cells, in which Ca^{2+}-dependent exocytosis is slow and regulated by slow Ca^{2+} spikes triggered by IP_3. Exocytosis occurs slowly in these cells largely as a result of a cytoskeletal meshwork that prevents translocation of LVs to neighboring plasma memberane. As mentiond above, this slow rate of exocytosis is consistent with exocrine cell function; for example, slow and relatively persistent exocytosis is thought to control the concentration of digestive enzymes in the gastrointestinal tract. Comparison between LV exocytosis in endocrine and exocrine cells thus sheds light on two ATP-independent steps of Ca^{2+}-triggered exocytosis: vesicle translocation and vesicle fusion-after docking.

Future studies based on the combination of various techniques of modern biology should aim to quantify separately these two steps of exocytosis in order to clarify further the underlying mechanisms. To this end, we are now applying multiphoton excitation of fluorophores and caged compounds, chromophore-assisted laser inactivation (CALI) of various SNARE-related proteins, and ultrastructural analysis with rapid freezing.

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